

STUDIES ON EGG SHELL PIGMENTATION IN THE DOMESTIC FOWL

GALLUS DOMESTICUS

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This thesis is presented for the degree of
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I declare that this thesis has been composed by myself.

The work presented has not been submitted to the
University in any other form

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Abstract

The results presented in this thesis confirm that in brown eggs laid by the domestic hen the intensity of colour depends on the amount of protoporphyrin-IX in the egg shell. This pigment which constitutes about 42% of shell porphyrin, is concentrated in the cuticle, the thin proteinaceous layer which covers the shell. Although the calcareous shell also contains pigment, its concentration, due to the larger mass, is vastly reduced in comparison to the cuticle, and therefore its contribution to the depth of colour is negligible. For this reason, the cuticle was used in this study to indicate the degree of shell colour intensity.

Variability in shell colour between eggs laid by individual hens is extremely large. In addition, in eggs from most hens, there is a decrease in shell colour as the laying cycle progresses. The loss of colour is attributed to a reduction in porphyrin content of the egg shell which is expressed in the cuticle. Overall, the lack of persistency in colour appears to be a less serious problem than was previously supposed, but it may still merit consideration in breeding programmes for dark shell colour. Furthermore, it was observed that darkly coloured eggs contained more shell than lighter ones, and it is suggested that selection for dark shells may be of practical value when wishing to improve shell strength.

While the cuticle was deposited on the shell at an even rate during the final stages of shell formation, within the shell gland pouch, this is not so for the pigment associated with it. By 3 hours before oviposition, 77% of the cuticle has been formed. Pigmentation, on the other hand is slow up to this point, after which time around 81% is added to the cuticle during the final 3 hours prior to oviposition. During this time, the depletion of shell gland tissue porphyrin corresponds to the uptake of porphyrin by the cuticle.

Oral administration of a porphyrin-inhibiting drug, Nicarbazin, reduced egg shell porphyrin content by more than 75%. The loss of pigment more closely corresponds to the magnitude in the reduction in shell gland tissue porphyrin, during treatment, than to the porphyrin content in the blood.

Finally, the biochemical processes of egg shell pigmentation and this relationship with cuticle formation are discussed.

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LITERATURE REVIEW

1.0 LITERATURE REVIEW

1.1 INTRODUCTION

The modern commercial laying hen is capable of producing over 300 eggs a year (Gilbert, 1972). The annual production of eggs in the United Kingdom is around 1,041 million dozen, from approximately 38 million hens and it has a value of over 500 million pounds. The egg industry is therefore of considerable commercial significance. It is particularly subject to market forces and one of the challenges it is faced with is to produce an egg which satisfies the demands of the retailer and consumer. Among the factors involved are albumen quality, yolk colour, shell strength and recently an increased emphasis on pathogenic bacterial content.

As a result of much research, the egg producer now has the knowledge to enable him to meet many of these requirements. In recent years another aspect of shell quality, namely shell colour, has become important to the consumer. Brown-shelled eggs currently dominate the markets of the United Kingdom, France, Ireland and Portugal, whereas white eggs are preferred in the United States, Australia, Austria, the Federal Republic of Germany, Switzerland and Spain (Anon, 1987).

Because of the demand for brown shelled eggs, shell colour has been increasingly emphasised in the sales promotion and marketing strategy of major egg retailers in the United Kingdom. It is therefore important for egg producers to be able to offer eggs with a shell colour acceptable to the consumer.

Two of the main problems which exist are variability in colour from egg to egg and decline in egg shell colour as the hen ages. In the current market, both problems are of considerable economic importance.

Much variation in shell colour also exists between both strains of hen and between individual flocks, and it is recognised that as the birds age, the situation worsens. Such variation constitutes a problem because there appears to be a consumer preference for eggs with a consistently dark brown shell colour. Unfortunately in past years there has been little interest in research leading to a better understanding of how shell pigmentation varies with age and strain of bird. Recently, however, there has been a renewed interest in the pigmentation process, mainly arising from these commercial problems encountered by the large egg producers. However, sound research programmes are required to be established to provide a better understanding of the reasons for the variability of egg shell pigmentation in commercial strains of the domestic fowl.

There have been many studies directed towards understanding the process of shell formation, shell structure and the mobilisation and secretion of calcium around the egg. The segment of the avian oviduct which is responsible for egg shell formation was previously termed the uterus. However, the gland is in no way homologous or comparable with the mammalian uterus and the term shell gland pouch is now preferred (Nalbandov, 1976). An egg is retained within the shell gland during the entire period of shell formation (i.e. about 20-22 h). Following calcification of the egg shell at around 17-18 h (Talbot and Tyler, 1974), a final coating is deposited around the hen's egg shell. This coating, the cuticle, consists mainly of protein, and appears to be pigmented in the eggs from brown-laying strains. When this final coating and pigmentation of the egg shell are complete, oviposition occurs. Early studies by Fischer and Kogl (1923) and Fischer and

Lindner (1925) showed that avian egg shell pigments are secreted by the shell gland epithelial cell layer. Later, Tamura and Fujii (1965) confirmed these findings. Two cells types within this epithelial layer exist; the apical ciliated cells and the basal mucous cells (Richardson, 1935; Johnston et al., 1963 and Tamura and Fujii, 1966). The basal cells (Tyler and Simkiss, 1959; Baker and Balch, 1962) have been implicated in cuticle secretion and three forms of granules are histologically distinct within the cells (Tamura and Fujii, 1966b). Their numbers were shown to change in a cyclic form throughout the period of shell formation. Tamura et al., (1965) suggest the apical cells are involved in pigment formation, but both cell types autofluoresce during the later stages of calcification (Baird et al., 1975).

A study by Warren and Conrad (1942) indicated that in the domestic fowl (Gallus domesticus), pigment was deposited during the entire period of shell formation but the rate accelerated in the last few hours before oviposition. Turkey eggs were also shown to acquire their superficial pigment speckles near the time of oviposition (Warren and Conrad, 1942). Similar studies with Japanese quail (Coturnix coturnix japonica), showed that superficial pigment started to be deposited on eggs around 3.5 h before lay (Woodard and Mather, 1964). Pigments in the shell of the domestic hen were thought to be distributed evenly throughout the shell membranes, shell matrix and cuticle, whereas quail cuticle contained most of the egg shell pigment (Tamura and Fujii, 1967). Romanoff and Romanoff (1949) suggested that a relationship may exist between the degree of pigmentation and the deposition of cuticle on the egg shell. These forms of study on egg

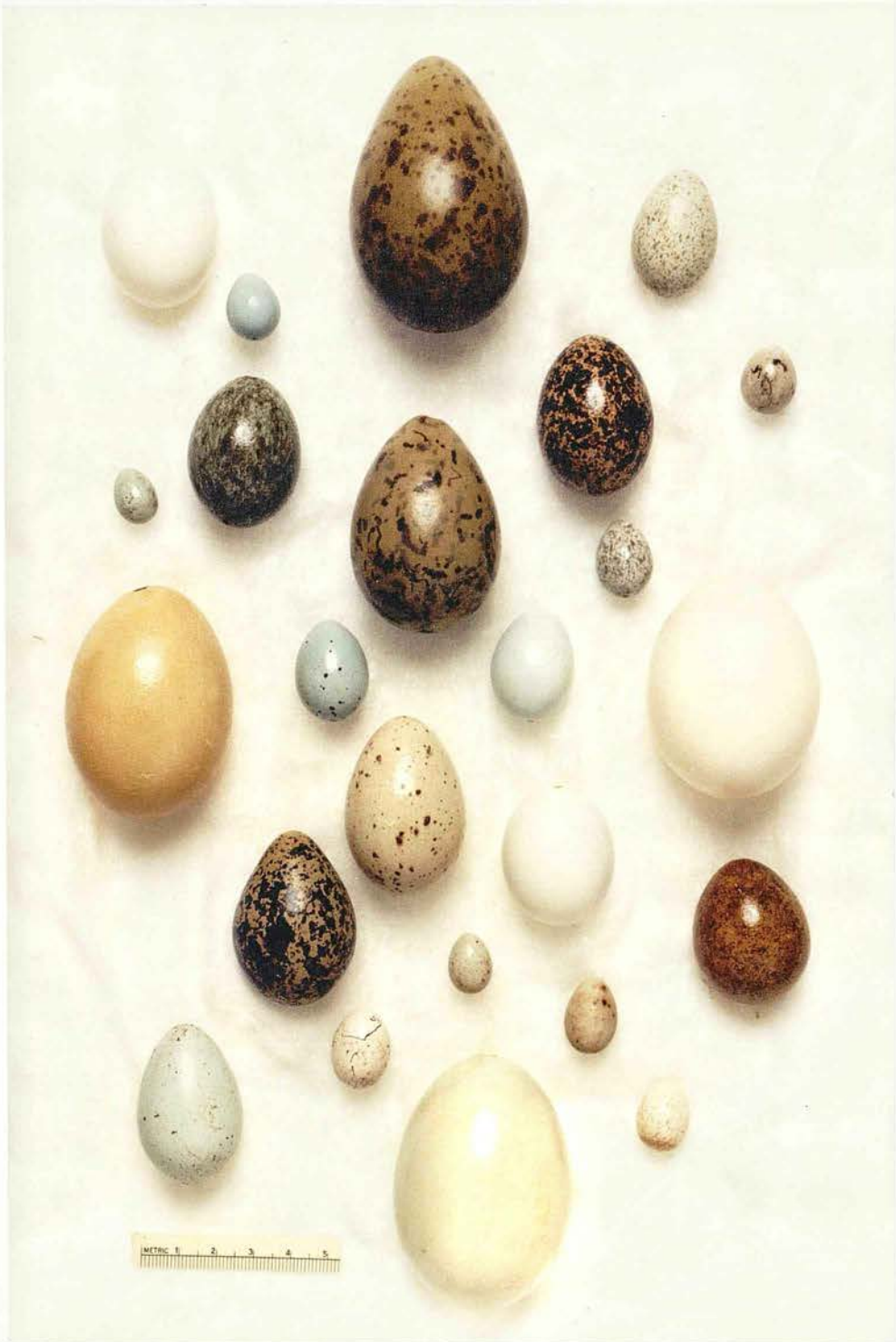


PLATE 1

A selection of wild birds eggs which demonstrates the wide range of colour and patterning.

shell pigment have interested biologists and zoologists for many years and much of the early work concentrated on the role that pigments play in the wild state. Plate 1 shows the wide range of colour and patterning on the eggs from wild species of birds. However, studies so far have not fully demonstrated the precise nature of the biochemical and physiological processes involved in the production, transfer and secretion of cuticle and pigment within the avian species.

1.2 EGG SHELL STRUCTURE AND FORMATION

Structure

The literature available on egg shell structure and formation is extensive (see Tullett, 1985 for a recent review) and most studies are exemplified by work on domestic fowl Gallus domesticus egg shells. It should, however, be recognised that the egg shells of other species are often specifically adapted to particular reproductive strategies. Variations may therefore exist between egg shells from different avian species. Studies using domesticated birds will nevertheless enhance our overall understanding of the mechanisms involved. With respect to shell colour, few studies have solely concentrated on the pigmentation process, an important activity taking place prior to the oviposition of the fully-formed egg shell.

It is necessary to firstly review the salient features of the egg shell and the activities taking place in the avian oviduct responsible for its formation, before discussing egg shell pigments in more detail.

The egg shell of the fowl weighs around 5 g. On its inner surface is a lining of two fibrous membranes which adhere to one and

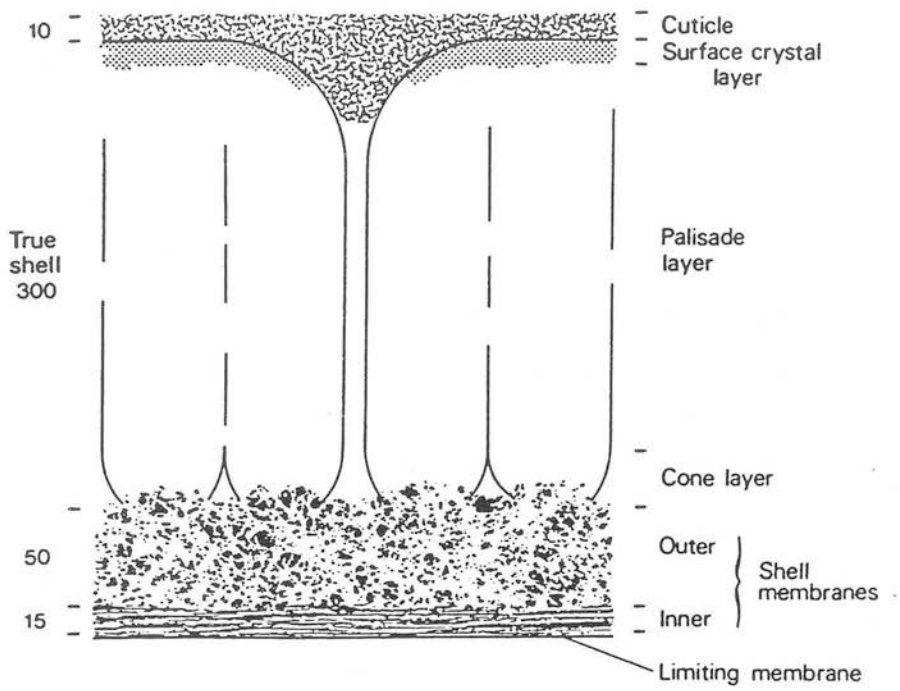


Figure 1.1 Radial section through a domestic fowl egg shell showing the main structural features. Numbers on the left of the diagram are thicknesses of each layer in μm .

other, forming a compound membrane lying around the albumen and ovum. The membranes contain approximately 2% ash (Wedral et al., 1974). On an ash free basis the membranes are about 95% protein (Simons, 1971; Baumgartner et al., 1978; Leach et al., 1981 and Crombie et al., 1981), 2% carbohydrate (Wedral et al., 1974) and 3% fat (Hasiak et al., 1978) in composition. Attached to the outer surface of the outer shell membrane are small masses of organic matter which act as 'seeding sites' for shell crystallisation to begin (Simkiss and Tyler, 1957; Baker and Balch, 1962). The mammillary cores (sites for calcium column development) consist of a protein-neutral mucopolysaccharide complex (Robinson and King, 1968).

The true shell is laid down on a matrix composed of fine fibrils of organic matter containing at least 70% protein with about 11% polysaccharide, including chondroitin sulphate A and B (Baker and Balch, 1962). A calcium-binding protein called ovocalcin has also been identified in the matrix fibres (Krampitz et al., 1980) and may be involved in the accumulation of the next layer, the true shell. This crystalline layer has a thickness of around 300-340 μm . It is composed of columns or cones of calcium carbonate, in the form of calcite and has a protein content of around 2%. Figure 1.1 shows the basic shell structure as exemplified by the domestic fowl. The final layer covering the external surface of the true shell has been termed the shell accessory layer (Board and Scott, 1980). The nature of these coverings varies between avian species. It may consist of a variety of white chalky coverings or different forms of organic deposits (Tullett et al., 1975; Board et al., 1977; Board and Perrott, 1979; Board et al., 1982). However, the most common form of accessory layer,

including the one found on shells of the domestic fowl is termed the cuticular layer. The cuticle of the domestic fowl shell is a thin (about 10 μm) coating which varies greatly in thickness. It covers the entire surface of most eggs and penetrates the shell pores in the form of a plug (Board and Halls, 1973). Simons (1971) described the cuticle as having a vesicular structure composed of spheres of organic material up to 1 μm in diameter. When viewed by scanning electron microscopy it has been said to have the appearance of dried mud (Becking, 1975). The cuticle has a characteristic amino acid pattern which distinguishes it from that of the shell matrix and membranes. Baker and Balch (1962) found the cuticle to be composed of about 90% protein and 10% carbohydrate. Wedral et al. (1974) estimated the protein composition to be between 85-87% with 3.5-4.4% carbohydrate, 2.5-3.5% fat and 3.5% ash.

Formation

The modern commercial laying hen may shed a mature follicle (the ovum) daily from the ovarian stalk. The ovum is then engulfed by the proximal end of the oviduct, the infundibulum. It spends about 15 mins there and the chalaziferous layer of the albumen is added around the ovum (Perry and Gilbert, 1979). The next and largest portion of the oviduct, the magnum, through which the ovum takes around 2-3 h to pass, secretes the concentrated albumen around the ovum (O'Malley et al., 1969; Wyburn et al., 1970). Secretion of the two shell membranes around the developing egg occurs rapidly (around 1 h) in the isthmus (Draper et al., 1972; Candlish, 1972 and Solomon, 1975). The egg then enters the tubular shell gland region where shell crystallisation

(mamillary core formation) takes place (Solomon et al., 1975; Davidson, 1973) and the "plumping" process begins during its 15-30 min passage through this region (Wyburn et al., 1973). Plumping involves the uptake of water, carbohydrate and ions from the shell gland fluid by the albumen, thus regulating the osmo-ionic composition of the egg. This uptake results in a distension of the shell gland wall which may act to stimulate the start of calcification. The egg then enters the shell gland pouch where shell deposition commences, slowly at first, but increasing rapidly to a constant rate until termination after 17-18 h (Talbot and Tyler, 1973). During calcification, blood flow to the shell gland represents 8.58% of the total flow (Boelkins et al., 1973) and has been reported by Hunsaker (1959) to be double the flow to the inactive shell gland tissue. These figures indicate that the blood is the most likely origin of calcium supply for shell mineralisation. Details of the transport system operating in this process are still unknown but Mongin and Carter (1977) give a general model of the process, and Nys and De Laage (1984) and Lundholm (1985) describe calcium binding proteins found in the shell gland cells. The cuticular layer and shell pigments are deposited during the final hours that the egg lies within the shell gland pouch. Again the details of these transfer mechanisms have yet to be described.

1.3 THE ROLE OF THE CUTICLE

The cuticle of the domestic fowl covers the entire egg shell and blocks the shell pores (see Figure 1.1) in the form of a 'plug' but due to its vesicular structure (Simons, 1971), the exchange of respiratory gases can still take place (Tullett and Board, 1976).

Respiratory gases must move freely across the shell, which therefore must be porous. The cuticle has a marked resistance to water and therefore protects the egg shell pores from flooding which would reduce the diffusion of respiratory gases. By preventing flooding of the pores, the cuticle also guards against penetration by microorganisms (Board and Halls, 1973 ; Board and Fuller, 1974; Board, 1982). Removal of the cuticle increases bacterial infection (Brown et al., 1966) and eggs devoid of a cuticle, with open pores, such as pigeon eggs, are more readily infected than eggs with an intact cuticle. A study by Vadehra et al. (1970) involved comparing the spoilage rates of normal eggs with those devoid of cuticle. The protection provided by the cuticle was estimated to last for 96 h or more, after oviposition. However, the theory that the cuticle equips the shell with a degree of mechanical strength to resist deformation was thought to be unfounded (Belyavin and Boorman, 1980). Nevertheless the cuticle does make a significant contribution to shell thickness when measured directly.

1.4 PIGMENTS IN AVIAN EGG SHELLS

1.4.1 The pigments

Sorby (1875), undertook the first studies on the nature of the pigments present in avian egg shells. Pigments from a number of avian species were subjected to spectroscopic analysis. The colour of egg shells was demonstrated to be mostly limited to either the red or green portion of the spectrum. The major pigment obtained from brown-shelled eggs was named "oorhodeine" by Sorby. Later Fischer and Kogl (1923) and Bierry and Gouzon (1939) recognised it as a porphyrin compound. "Ooporphyrin", as it was then referred to, was crystallised and later

its identity was established as the haemoglobin porphyrin, protoporphyrin-IX (see Figure 1.2a). Of more than 20 species studied, shells of all but one showed at least traces of this porphyrin. Earlier, Wicke (1858) studied the bluish-green pigment found in the shells of certain species, such as the carrion crow (Corvus corone) and the song thrush, (Turdus musicus). Krukenberg (1883) believed it to be identical to the bile pigment biliverdin. Lemberg (1934) and Tixier (1945) finally confirmed Krukenberg's study by isolating biliverdin-IX in a crystalline form. Later, Kennedy and Vevers (1973) showed that the major pigments in the blue-green shell of the Araucano fowl (a Chilean mutant of the domestic fowl) were biliverdin-IX (see Figure 1.2b), its zinc chelate and protoporphyrin-IX. Poole, (1966) demonstrated that the two pigments in quail eggs are protoporphyrin-IX and biliverdin-IX. By quantifying the pigments protoporphyrin-IX and biliverdin-IX in quail eggs, Lucotte et al. (1975) were able to construct polychromatic maps from each of seven phenotypes. It was found that protoporphyrin-IX was the more variable of the two pigments and that biliverdin-IX concentration remained fairly constant in eggs from an individual bird and from the eggs of quails from within a phenotype (Lucotte et al., 1975).

With (1973), using chromatographic techniques, showed that there were significant amounts of the other porphyrins, uroporphyrin and coproporphyrin in addition to protoporphyrin in the brown shells from an undefined breed of domestic fowl.

A more extensive study was carried out by Kennedy and Vevers (1975) who surveyed the shell pigments of 106 avian species. Their findings indicate that the pigments present in egg shells are proto-

protophyrin-IX, biliverdin-IX and its zinc chelate, with occasional traces of coproporphyrin-III. Almost 50% of the species examined by Kennedy and Ververs (1975) contained only protoporphyrin in their shells. No pigments were found in the white egg shells of the fulmar, imperial pigeon, dipper, roseate cockatoo and ring-necked parakeet. Some apparently white egg shells contained only protoporphyrin, e.g. the white stork, Barbary dove, Scops owl and roller. Others contained both protoporphyrin and biliverdin, e.g. shells from the black-footed penguin, Humbolt's penguin, mandarin duck and wood pigeon. The species studied belong to widely separated families and there appears to be no obvious correlation between genetic classification of a species and its egg shell pigment (Kennedy, 1975).

The genetics of shell colour has interested many workers in the past. When white-egg layers are crossed with brown-egg layers, the F1 generation lay eggs intermediate (tinted) between the two parental types. This factor has indicated that shell colour is determined by multiple factors (Punnet and Bailey, 1920). Genetic transmission of shell colour and pattern appears to be hereditary (Lucotte et al., 1975) and that the factor is transmitted through the female of the species.

1.4.2 The function of the pigments

Modern reptiles have white to off-white egg shells (Solomon, 1987). Ancestral avian progenitor reptiles probably by inference also produced white eggs. The eggs of most primitive living birds:- grebes, petrels, shearwaters, ostrich and cormorants are white or near-white. Colouration and pigmentation therefore appeared during avian evolution

and must confer some selective advantage, which may however, be modulated by geographical, behavioural or nest site differences between species. Welty, (1962) suggested avian egg shells firstly acquired the ground colour and secondly the markings throughout the evolutionary process. However, the functional value of pigmented eggs has been difficult to demonstrate with the following putative functions being proposed.

Crypsis

Animal colouration and patterning was thought, by early workers, to offer camouflage protection from visually hunting predators (Poulton, 1890; Cott, 1940; Portmann, 1959). This concept, however, has not been fully demonstrated with pigmented egg shells and the results of many experiments have provided conflicting evidence. Tinbergen (1963, 1967) presented quantitative evidence of the importance of natural egg shell patterning in comparison with white eggs in the face of avian predation. Similarly, Montevecchi (1976) placed cryptic and white eggs in meadows visited by avian predators and found that cryptic eggs suffered less predation. More recently, Westmoreland and Best (1986) showed the value of cryptic colouration, in the field, using mourning doves (Zenaida macroura), both when incubation was interrupted and continual. Bertram and Burger had similar results when they placed white vs brown Ostrich (Struthio camelus) eggs on bare ground. In contrast, Kruijt (1958) found that crypsis did not reduce predation by Herring Gulls (Larus argentatus) on artificial nests.

Recognition

Further conflict exists when highly camouflaged eggs are laid in concealed nests which removes the need for protective colouring (Romanoff and Romanoff, 1949). It has been proposed, however, that egg shell colouring intensifies the maternal instinct which attracts the brooding bird to the nest and to incubate the eggs. Similarly, Harrison (1975) suggested that the very variable pattern and colour of guillemot eggs, normally found in large colonies allow the parent to recognise its own egg. Further support for the recognition theory came from Lack (1958) who suggested the white colour of eggs evolved as an aid to recognition in dimly-lit places. Most, if not all, white eggs are produced by birds laying in dark places, e.g. holes, and by birds which cover their eggs.

Heating and solar gain

Early workers (Darwin, 1868; McAlldowie, 1886 - cited by Romanoff and Romanoff, 1949) suggested that shell pigmentation is an adaptation to shield the developing embryo from sunlight, and in general, eggs of tropical species tend to be darker than those from cooler climates. Conversely, Montevecchi (1976) noted that uniformly light-coloured egg shells provide far greater solar shielding than do darkly pigmented eggs. It is thought that the pigmentation and patterning of eggs may play a functional role in embryonic development (Pettinghill, 1970). Solar radiation impinging on the yolks via the egg shell may increase hatchability in darker eggs in comparison with unpigmented ones. Alternatively, the avian embryo is injured or killed by temperatures more than a few degrees above normal incubation values

(Moreng and Shaffner, 1951; Drent, 1970). Field investigators have found that overheating by solar radiation is a serious threat to the survival of unattended eggs (Drent, 1970). Thus, selection due to predation appears to favour pigmentation whereas selection resulting from solar heating appears to oppose pigmentation (Bakken et al., 1978). These workers demonstrated that this conflict is minimised by egg shell pigments that absorb only visible radiation. Cryptic pigmentation does not significantly reduce the extremely high intrinsic near infra-red reflectance of egg shells. Solar heating is thus minimised, because half of the incident solar energy is in the near infra-red range. However, the white-cream coloured ostrich egg shell appears to contradict the adaptive theories of open nesting birds, which leave unattended eggs. Bertram and Burger (1981) reduced the rate of predation by staining the eggs brown, but untreated eggs, exposed to the sun retained a slightly cooler interior.

Warning colouration

An interesting series of papers were published by Cott (1948-54) on the palatability of birds eggs to various mammals. Among the conclusions was the suggestion that those species whose nests are most vulnerable to attack by predators have the most distasteful eggs. Further, such eggs often have a distinctive or conspicuous colour which has evolved as a protective adaptation against enemies. Cott proposes that this is an example of true aposematism in accordance with the classical theory of animal warning colouration.

The pigmented avian egg shell, therefore appears, in many instances to be a unique surface with its combination of cryptic visual colouration and thermoregulatory near infra-red reflectance.

Colouration of the domestic fowl egg shell

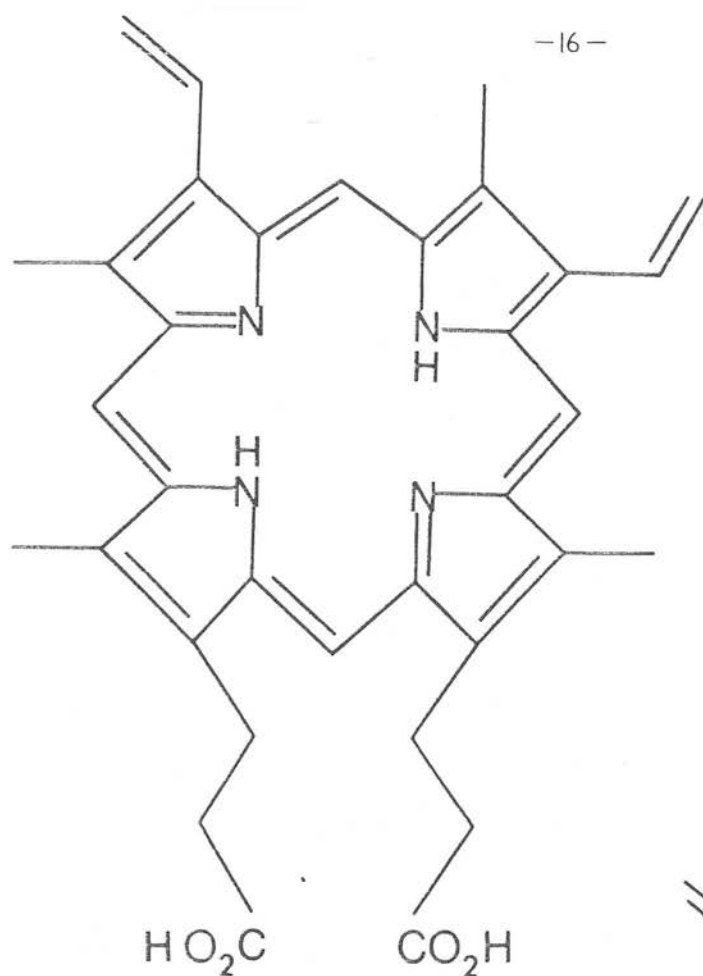
The domestic fowl (Gallus domesticus) is unusual in that, many members of the species lay conspicuous white eggs, although incubation is mostly continuous, with the hen rarely leaving the nest (B. Hughes, personal communication). Selective pressure for egg crypticity is probably weak. Westmoreland and Best (1986), studying Mourning Dove eggs, which are white and are continuously incubated, suggested that the eggs may have originally been cryptic. They have lost colour because continuous incubation eliminated selection for the trait. Conversely, they may have evolved constant incubation to cover their conspicuous white eggs. However, Westmoreland and Best (1986) thought the former was most likely with crypticity offering an extra degree of protection during periods in which the birds are forced to leave the nest-site to escape predators. A similar explanation may partly account for fowl laying conspicuous or cryptic eggs, although they have been domesticated for some time.

1.5 BIOSYNTHESIS AND PROPERTIES OF EGG SHELL PIGMENTS

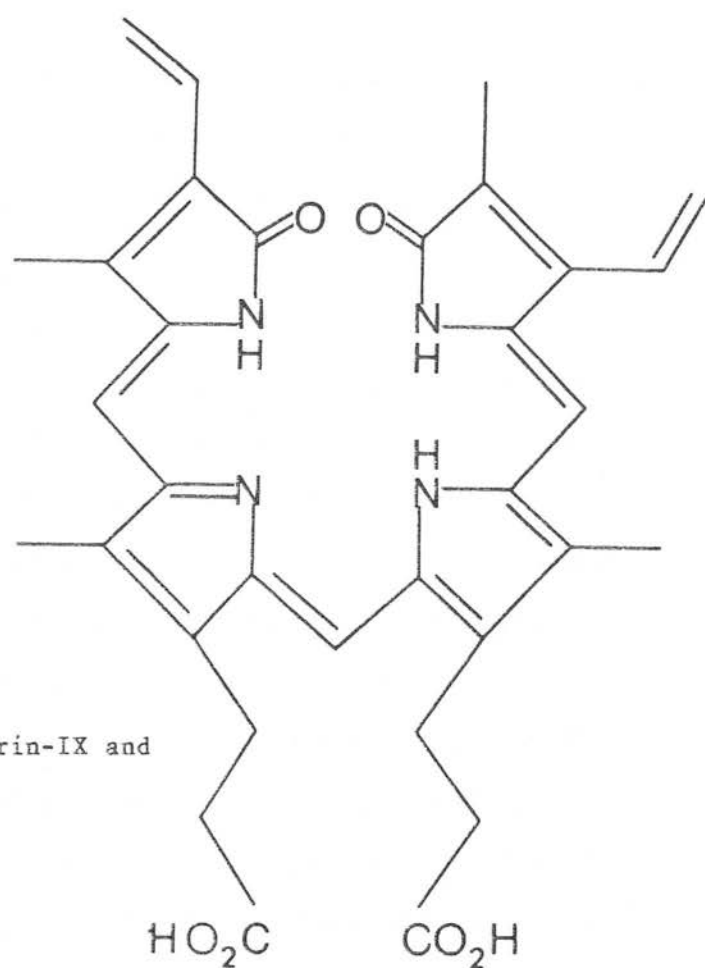
1.5.1 The Porphyrins

Porphyrins were first mentioned in the literature around 1840 and the first pure porphyrin was isolated at the turn of the century. The full structure was finally established by Fischer and Kogl (1923), while Fischer and Orth (1937) synthesised the first porphyrin compound.

Porphyrin derivatives play an important role in the biochemistry of all living systems, indeed they have been called the pigments of life (Battersby, 1985). The porphyrin structure is found in pigments such as the chlorophylls and haem. Porphyrins and their



(a) Protoporphyrin - IX



(b) Biliverdin - IX

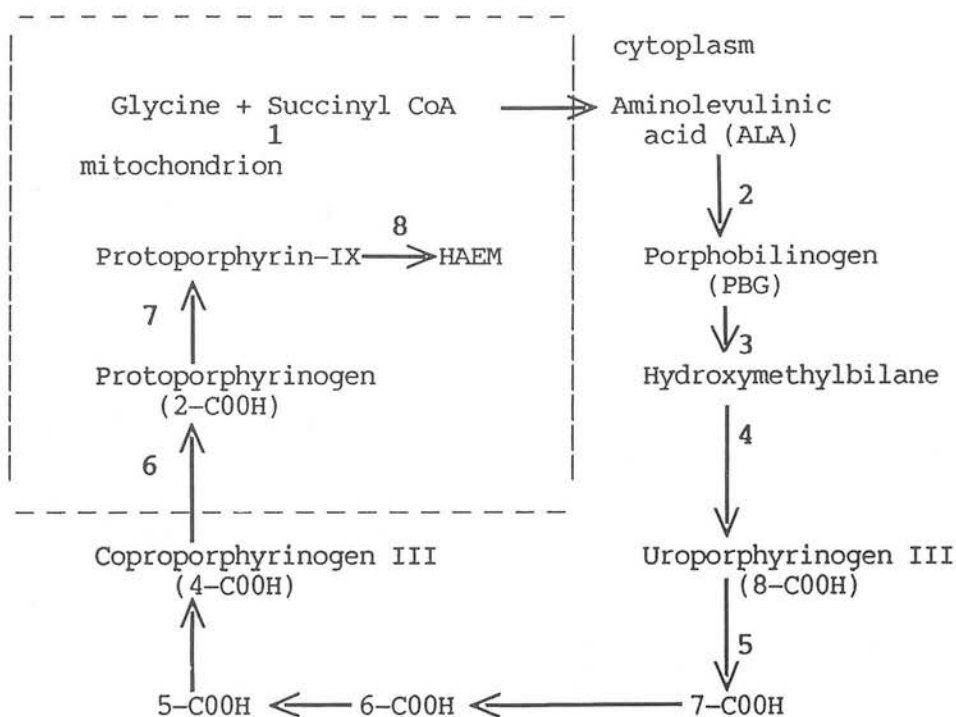
Figure 1.2 The structure of protoporphyrin-IX and biliverdin-IX.

derivatives are also present in a wide variety of other biocatalysts, e.g. cytochromes, vitamin B₁₂ and prosthetic groups of enzymes whose biosynthesis was likely to be contemporary with the appearance of life on earth (Simionescu et al., 1978).

Porphyrins comprise cyclic tetrapyrrole structures and are all related to the parent compound porphine. In nature, protoporphyrin-IX (Figure 1.2a) is the most common form of the fifteen possible isomers among four different possible combinations of the side chains on the porphyrin nucleus. In porphine, hydrogen atoms replace the methyl, vinyl and propionic acid side chains. Incorporation of isotopically-labelled intermediates showed that all the carbon and nitrogen atoms of the porphyrin ring are derived from glycine and succinic acid. Porphyrin synthesis involves the formation of colourless chromogens, the porphyrinogens such as uroporphyrinogen and coproporphyrinogen. The porphyrinogens are the primary excretion products of the pathological disease in man and animals called porphyria. These substances contain methylene bridges ($-\text{CH}_2-$) instead of methyne bridges ($-\text{CH}=\text{}$) between the pyrrole nuclei found in porphyrins. Porphyrinogen can then be auto-oxidised to the corresponding coloured uroporphyrin and coproporphyrin. Uro'gen-III, (uroporphyrinogen III) is, however, the precursor to all pigments such as haem, the chlorophylls and vitamin B₁₂ (Battersby, 1985). Figure 1.3 shows the haem biosynthetic pathway in which the egg shell pigment protoporphyrin-IX is formed.

The spectrum of most common porphyrins in neutral solvents consists of four relatively sharp bands, increasing stepwise in intensity from band I, in the region of 620 nm to band IV, near 500 nm. Between bands I and III a very small band, Ia occurs in some porphyrins

Figure 1.3. Haem biosynthesis



- Enzymes:
- 1 ALA synthetase (EC 2,3,1,37)
 - 2 ALA dehydratase (EC 4,2,1,24)
 - 3 PBG deaminase (EC 4,3,1,8)
 - 4 Uroporphyrinogen co-synthetase (EC 4,2,1,75)
 - 5 Uroporphyrinogen decarboxylase (EC 4,1,1,37)
 - 6 Coproporphyrinogen oxidase (EC 1,3,3,3)
 - 7 Protoporphyrinogen oxidase (EC 1,3,3,4)
 - 8 Ferrochelatase (EC 4,99,1,1)

(Falk, 1961). This type of spectrum in the visible region is called the "Soret" band and is a characteristic feature of porphyrins (Soret, 1883).

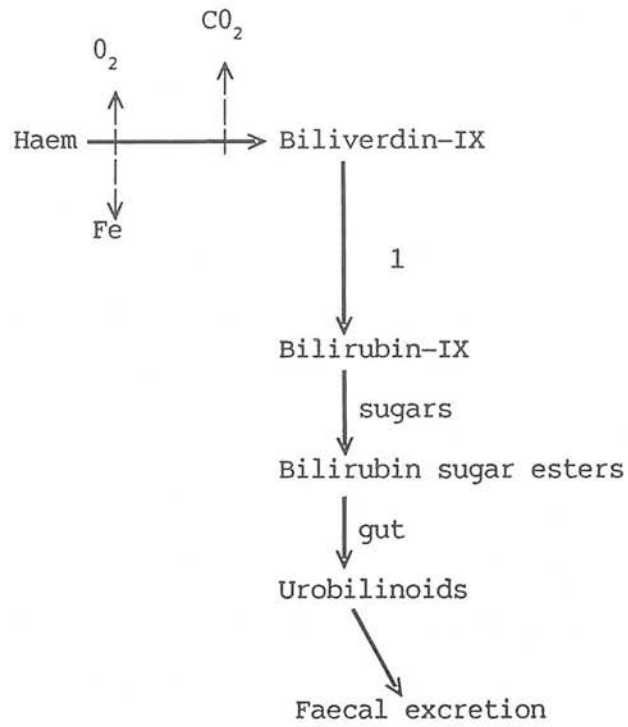
On exposure to light at approximately 400 nm, porphyrins emit a bright orange to red fluorescence. This emission is a characteristic property of the porphyrins since orange-red fluorescence from non-porphyrin sources is rare. This fluorescent property provides a very sensitive method for detecting small amounts of porphyrin, provided it is in solution. The fluorescence of solid porphyrins is generally quenched or reduced, but porphyrins absorbed on talc, alumina, cellulose, calcite or protein (such as in egg shells) fluoresce brightly. Porphyrin fluorescence is best observed in dilute HCl solution: as little as 0.1 $\mu\text{g/ml}$ can be detected by the naked eye when the solution is irradiated by an ultra violet light source. Using a spectrofluorimeter, as little as 100 pg/ml can be detected in acid solution (Lennox, 1979).

1.5.2 The bile pigments

In contrast to the porphyrins, bile pigments are open chain tetrapyrroles. They are derived in nature by oxidative degradation and ring-opening of the prosthetic groups of haemoproteins (Hudson and Smith, 1975).

The principal sites of haemoglobin breakdown are the reticulo-endothelial cells of spleen, bone marrow, liver and to a smaller extent, the kidneys. Rupture of the blood pigment, with the loss of the mesocarbon of the methyne bridge (as carbon monoxide), produces biliverdin (Figure 1.2b). In man and mammals, biliverdin is reduced,

Figure 1.4. The metabolic degradation of haem to bile pigment in man and other vertebrates



Enzyme: 1 biliverdin reductase

giving bilirubin, the reduction is catalysed by biliverdin reductase. Figure 1.4 shows the metabolic degradation of haem to bile pigment in mammals and other vertebrates. However, the system responsible for haem catabolism in vivo has not been fully elucidated.

A review by Schmid and McDonagh (1979) gives a detailed description of the alternative catabolic systems.

Biliverdin is the primary green pigment (max 380 and 666 nm) found in the egg shells of certain birds including the song thrush Turdus musicus, carrion crow Corvus covone and the Araucano fowl (Kennedy and Vevers, 1975).

The two principal pigments of egg shells, protoporphyrin-IX and biliverdin-IX, have different origins, despite their chemical similarities. The porphyrins are likely to be synthesised de novo in the cells in which they occur. On the other hand, biliverdin is probably derived from erythrocyte catabolism.

1.6 THE ORIGIN OF EGG SHELL PIGMENTS

All animal cells have the capacity to synthesise porphyrin. The biosynthetic pathway starts with the two basic precursors, succinyl CoA from the tricarboxylic acid cycle and glycine, which combine by condensation (see Figure 1.3) within the mitochondrial fraction (Moore, 1980). It then proceeds through a series of reactions forming intermediate precursors, and finally with the insertion of iron into the porphyrin ring to form haem. Indeed, many studies have utilised suspensions of avian erythrocytes to investigate the intermediates of the porphyrin biosynthetic pathway. However, within the class Aves there is a high proportion of incomplete synthesis producing large

quantities of porphyrin (mainly protoporphyrin-IX) which is utilised to colour their egg shells. Nevertheless the precise location of porphyrin synthesis for this purpose within the avian, is obscure and two schools of thought have developed concerning the origin of porphyrin synthesis; that porphyrins are formed within the erythrocytes and are transferred to the shell gland and deposited on the egg shell via the surface epithelial cells; the other possibility is that porphyrins are synthesised within the shell gland tissue itself and again transferred to the egg shell via the epithelial cells. The proposals for each of the above systems are discussed in the following sections.

1.6.1 Blood-derived

Blood flow through the shell gland during the calcification process supplies calcium to the shell at a rate of 1.67 mg-2.0 mg per minute (Sturkie, 1986). A proposal which is also made is that egg shell porphyrins are also blood-derived and are transferred to the egg via the shell gland epithelial cells. Kennedy and Vevers (1973) considered that the porphyrins were derived from erythrocytes which are known to synthesise porphyrin (Dresel and Falk, 1954; Granick and Mauzerall, 1958). Their arguments were based partly on histological studies by Giersberg (1921) who held that pigments were derived from disintegration of erythrocytes in the mucous layer of the shell gland. In their 1973 paper, Kennedy and Vevers discussed theories of the pigmentation process, especially in relation to calcification, but no experimental evidence was given to support these ideas.

1.6.2 Shell gland-derived

On examination of the shell gland under an ultra-violet light, a bright red fluorescence is revealed, confined to its epithelial layer (Tamura et al., 1965). The surface epithelial cells are pseudo-stratified columnar with the nuclei arranged in two layers. Two cell types are recognised. Deeply placed nuclei are located in the basal cytoplasm close to the plasma membrane in one type and more superficially arranged nuclei in the luminal half of the cytoplasm of the others. Cells with basal nuclei are termed basal cells (Tyler and Simkiss, 1959; Baker and Balch, 1962) and those with superficial nuclei, the apical cells. Both types of cells have been implicated in the production of cuticle. Tamura and Fujii (1965, 1966, 1967b) found small pigment granules containing porphyrin in the apical cells together with larger Periodic Acid-Schiff-positive granules. The abundance of both types of granules increased progressively until cuticle deposition; thereafter their numbers declined rapidly. These authors concluded that the cuticular porphyrins are derived from the small pigment granules. A schematic section of the shell gland mucous epithelium at various stages during egg formation in quail is shown in Figure 1.5.

Chromatographic analysis by With (1973) of egg shell pigment from an undefined breed of domestic fowl demonstrated the presence of protoporphyrin-IX and significant quantities of other porphyrins, viz; uroporphyrin, pentacarboxylic porphyrin and coproporphyrin. With concluded that synthesis of porphyrin probably takes place in the shell gland, and such a mixture could not be derived solely from haem degradation. Solomon (1987) considered that the non-fluorescing

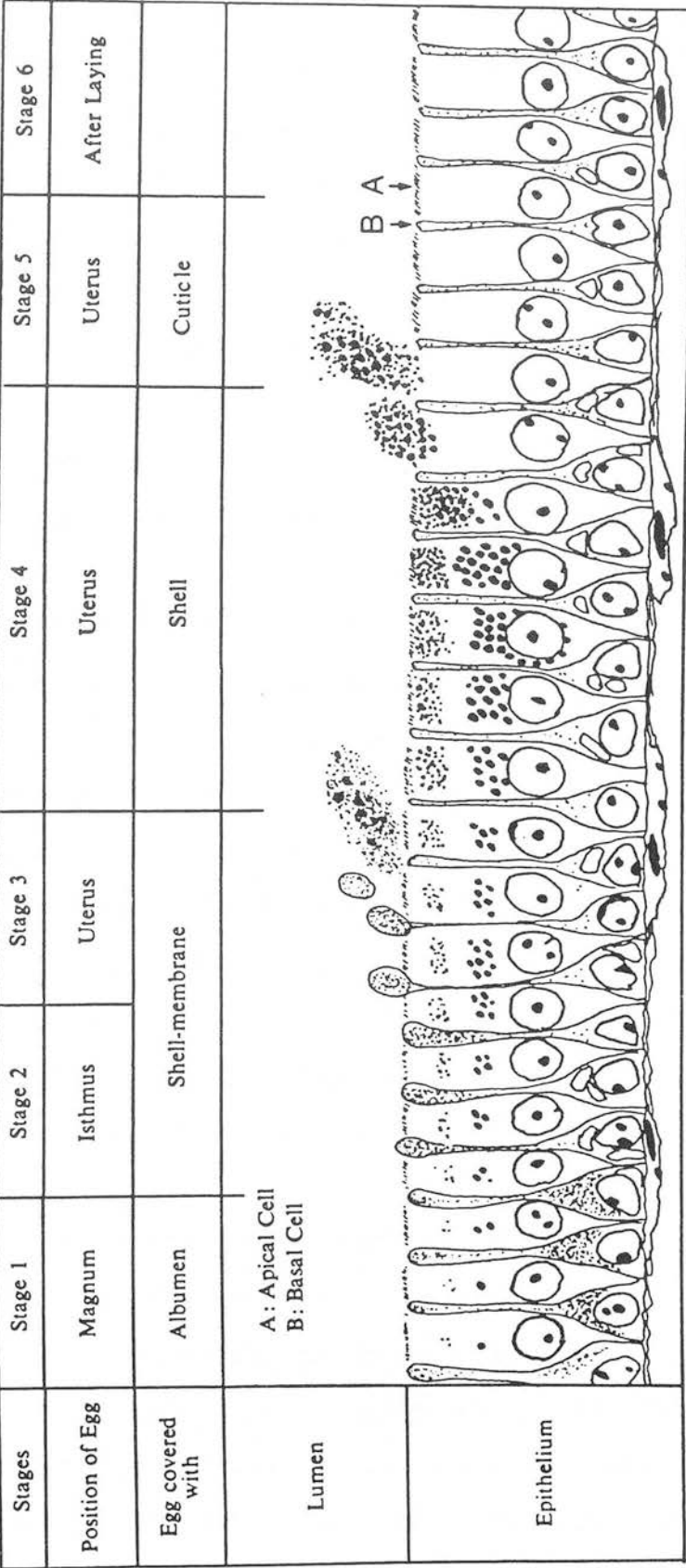


Figure 1.5 Changes in the secretion of the shell gland mucous epithelium of the Japanese quail (Tamura and Fujii, 1966).

tubular gland cells of the shell gland may be implicated in the production of the colourless porphyrinogens. These substances could then be transferred to the epithelial cells where they are auto-oxidised to the porphyrin pigments. These postulations were based on the observation that tubular gland cells possess a fluctuating population of mitochondria (which are probably the sites of cellular porphyrin synthesis) throughout egg formation and that the epithelial cells are poorly equipped in this respect.

The presence of enzymes of the porphyrin biosynthetic pathway has been demonstrated in shell gland tissue. Shell gland homogenates from domestic fowl (Polin, 1957) and Japanese Quail (Poole, 1966) can synthesise porphyrins from 5-aminolevulinic acid (ALA) in vitro. Stevens et al. (1974) showed that ALA-synthetase and ALA-dehydratase were present in shell gland tissue and that their levels varied according to oviducal activity. Lucotte et al. (1975) has also demonstrated porphyrin synthesis with quail tissue, in vitro and that levels of protoporphyrin increased three-fold with a pigmented egg in situ. This work, together with that of Yamada (1972) lends support to the hypothesis that the avian shell gland may be the site of biosynthesis of egg shell porphyrins.

1.7 CHANGES IN EGG SHELL PIGMENTATION

Obvious differences in egg shell colour intensity can exist between strain of commercial laying hens. However, these differences are normally small and probably of little economic importance. Persistence of colour with age is likely to be more important, but this aspect has not been fully investigated, with mainly casual acknowledgements that shell colour deteriorates with the ageing flock.

Disease, for example, that caused by the infectious bronchitis virus causes a marked increase in the incidence of pale-shelled eggs (Cooke, 1986) and it has been suggested this may be a result of the virus's damaging effect upon the shell gland's epithelial cells.

Similar sharp declines in colour have been observed when laying hens have inadvertently been administered the coccidiostatic drug Nicarbazin, which causes almost complete loss of shell colour.

A variety of surface defects as well as egg shell "pimpling" have become more obvious due to the prominence of brown eggs in the U.K. market. These aspects have been considered with reference to shell colour in a review; see Lang and Wells (1987).

BACKGROUND TO AND OUTLINE OF EXPERIMENTAL PROPOSALS

2.0 BACKGROUND TO AND OUTLINE OF EXPERIMENTAL PROPOSALS

A research programme was devised to investigate a number of points which arose from an existing problem, - the decrease in shell colour intensity as the laying hen ages. Later studies were designed to study the processes of shell pigmentation in greater detail. The research work was intended to investigate the following areas:-

1. The effect of age on egg shell colour intensity, using reflectometry, in eggs from a commercial laying strain (ISA Brown) of domestic fowl. Further studies aim to measure the effect that other physical factors may play on influencing shell colour.
2. Identification of the portion of the hen's egg shell which is responsible for expressing colour.
3. The development of a technique to isolate an individual cuticle from the egg shell to utilise in chemical investigations on shell colour.
4. The development of methods to estimate the mass of an individual cuticle, and measure the amount of pigment present within the egg shell.
5. Investigation into the relationship between some physical measurements on egg shell pigmentation and chemical analysis on cuticle and shell.
6. Study of the effect of age on the deposition of egg shell cuticle and pigment.
7. Determination of the quantities and rates of egg shell cuticle and pigment formation prior to oviposition.

8. The investigation of the pigmentation process with the use of a pharmacological compound which reduces the degree of shell colour.
9. Comparison of the egg shell cuticle and pigment content in eggs from a range of domestic poultry.

METHODS AND MATERIALS

3.0 METHODS AND MATERIALS

3.1 MANAGEMENT OF EXPERIMENTAL HENS

Hens of a commercial egg-laying strain (ISA Brown Hybrid) were individually caged with food (PRC diet, Bolton and Blair, 1974) and water available ad libitum. The birds were maintained under a lighting regime of 14 h light and 10 h darkness with the positioning of the lighting schedule varying according to the desired time which oviposition was required, within a day, for each individual experiment (see Section 4.5). Each cage was fitted with a custom built automatic egg recorder which scans the cages every 30 sec. to obtain a record of oviposition for individual members of the flock. Hens used throughout this investigation were normally aged between 25 and 35 weeks at sacrifice or the beginning of the study. Hens were sacrificed by an intravenous overdose (2 ml) of sodium pentobarbitone (Expiral, Ceva, Southampton).

3.2 SEPARATION OF CUTICLE FROM EGG SHELL

Preparation of ethylenediaminetetra-acetic acid (EDTA): normally 5 l of EDTA solution was prepared. 286.2 g of EDTA (disodium salt) was slowly added to 2.5 l of deionised water and heated gently on a magnetic stirrer hot plate until dissolved. The mixture was then made up to 5 l with deionised water and 37 g of sodium hydroxide pellets added to give a pH of 7.6-8.0 and a final concentration of 0.156 M.

The intact whole egg was firstly washed with deionised water to remove adhering debris. It was placed in a 100 ml plastic beaker and approximately 50 ml (depending upon egg size) of 0.156 M EDTA solution,

pH 7.6-8.0, added to completely submerge the egg. The egg was left to soak for 2.5 h, then the EDTA was decanted and the egg carefully transferred to a plastic filter funnel. Cuticle removal was accomplished by directing a fine jet of deionised water on to the egg shell, resulting in the cuticle peeling away from the underlying shell. Later a check for complete removal of cuticle was carried out. The egg was placed in a 1% aqueous solution of Edicol Pea Green H stain for 2 min (Ball et al., 1975) and washed with deionised water. Complete removal was indicated by the absence of intense green stain on the egg shell surface. The suspension of cuticle in water was recovered by filtering through a glass fibre circle, Whatman, No.54 GF/A, diam. 9 cm. The cuticle on GF/A was left overnight to dry under a darkened fume cupboard and stored in a shaded dessicator over a saturated solution of calcium chloride (hexahydrate) until required.

3.3 ACID HYDROLYSIS OF EGG SHELL CUTICLE

Reagent: 1 vol. constant boiling point hydrochloric acid (20.24%)

to 3 vol. formic acid (98%).

1. The dried cuticles (see section 3.2 for a description of cuticle separation) on GF/A support were folded into a small pellet and placed into 50 ml Teflon Oak Ridge centrifuge tubes (Nalgene Company).
2. Nitrogen gas was bubbled through the reagent mixture for 10 min.
3. To each tube 20 ml of the reagent were added using an Oxford Pipettor and the tubes gassed with nitrogen for 3 min.

4. Air was displaced from the cap of each tube with nitrogen gas and the tube tightly sealed.
5. The tubes were shaken vigorously for 30 sec. to disintegrate the GF/A support and adhering cuticle.
6. The caps were secured tightly and the tubes placed into a preheated oven at 94°C. Section 4.3.5 demonstrates why an enclosed hydrolysis system was chosen.
7. After 3 h the tubes were withdrawn, and again shaken vigorously for 30 sec. while covering each tube with a cloth for protection from possible leakage.
8. The tubes were returned to the oven and heated for a further 19 h.
9. When the tubes were cool, the mixture was centrifuged at 260 g for 15 min at room temperature in a Damon/IEC Division CRU-500 centrifuge.
10. The supernatants were filtered to optical clarity through a Whatman No. 1 filter paper and stored in glass vials in darkness until required for assay.

All stages of the procedure are carried out under subdued light to reduce photodegradation of the porphyrin.

3.4 PROTOPORPHYRIN-IX ESTIMATIONS

3.4.1 Egg shell cuticle (Method 1)

A qualitative check for the presence of porphyrin in the cuticle extract (the procedure is shown in Section 3.3) was routinely carried out by irradiating the solution under a u.v. light source. The presence of an intense orange-red fluorescence indicated porphyrin in solution.

Prior to the porphyrin assay the spectrum of the cuticle extract was established using a Unicam SP800 variable wavelength spectrophotometer, situated in a darkened room. A check for the characteristic absorbance maximum of protoporphyrin-IX, at 408 nm in the Soret region, confirmed its identity in the extract.

Porphyrin was estimated spectrophotometrically (Sveinsson, Rimington and Barnes, 1949; With, 1955). A small aliquot of the hydrolysate extract was placed into a 2 mm diam. glass cuvette and the absorbance read at the wavelengths, 380, 408 and 430 nm with a Pye/Unicam SP500 Series 2, u.v. spectrophotometer. Readings were made against a blank of GF/A filter and the reagent mixture treated as before.

3.4.1.1 Egg shell cuticle (Method 2)

The cuticle was separated from the egg shell as before and collected on a GF/A filter. The filter was blotted dry and excess filter carefully trimmed from around the cuticle sample. It was placed into an Oak Ridge centrifuge tube containing 20 ml of the HCl-HC00H (5%) reagent mixture. The tubes were gassed with nitrogen for 3 min, capped and shaken vigorously for 30 sec. and left to stand overnight in a darkened cupboard. After standing, the extracts were again vigorously shaken and centrifuged at 260 g at room temperature for 15 min. The supernatants were filtered as before through a Whatman No. 1 filter and the spectrum checked and porphyrin content estimated spectrophotometrically at the wavelengths 380, 408 and 430 nm as previously described against a blank of GF/A and reagent mixture.

Calculation of protoporphyrin-IX in solution for Methods

1 and 2 above:-

$$2 A_{408} - (A_{380} + A_{430}) \times 1.28 \times \text{vol. HCl-HC00H (ml)} \\ = \mu\text{g protoporphyrin-IX/cuticle}$$

where A = absorbance at each wavelength

The factor 1.28 was derived from the absorbance of pure protoporphyrin-IX in the solvent system employed (Heller et al., 1971)

3.4.2 True shell and membranes

The cuticles were removed from the eggs as before by soaking in EDTA solution for 2.5 h, normally with a bulk preparation of eggs. Each egg was broken, the contents washed out with deionised water and left to dry overnight in a darkened fume cupboard. The egg shells were weighed, crushed in a beaker, and 40 ml of the reagent mixture HCl: HC00H (5%) added to each sample. Enough HCl (20.24%) was added to each beaker (depending on shell weight) to neutralise the calcium carbonate. The mixtures were left to stand overnight then filtered through a Whatman No. 1 filter. The residues were washed (X3) with more reagent mixture and the filtrate made up to 100 ml in a volumetric flask. A check for complete porphyrin extraction was made by irradiating the sample under a u.v. light source. The absence of a porphyrin fluorescence confirmed complete extraction. Absorbances of the filtered extracts were measured at the wavelengths 380, 408 and 430 nm and against a blank of the acid mixture.

Calculation:-

$$2 A_{408} - (A_{380} + A_{430}) \times 1.28 \times \text{vol. HCl-HC00H (ml)} \\ = \mu\text{g protoporphyrin-IX/true shell}$$

3.4.3 Shell gland pouch and oviducal tissue

Reagents: acetone 1 vol: to ethyl acetate 9 vol.

formic acid (98%): to diethyl ether 9 vol.

1.5 M HCl

0.156 M NaCl

The method by Heller et al. (1971) to measure blood proto-porphyrin-IX was modified to estimate oviducal porphyrin. The whole oviduct, when required, was removed from the hen by cutting above the infundibulum and immediately before the entrance to the vagina. It was washed with ice-cold isotonic saline (0.9%), weighed and returned to a bed of ice. The shell gland pouch was separated from the oviduct by cutting below the isthmo-uterine junction. The gland was weighed. A cut was then made along the longitudinal axis with small surgical scissors and a portion (approx. 5 g wet weight) of tissue was cut from the inner lumen and then shredded and homogenised into fine pieces using the surgical scissors. Samples, 3 x 1 g wet weight, of homogenised tissue were accurately weighed and placed into 50 ml chilled plastic centrifuge tubes. Likewise, other portions of the oviduct were individually weighed and treated in the manner described in this section. To each tube 2 ml of acetone: ethyl acetate (1:9) solution were added and the tubes vigorously shaken for 30 sec. Formic acid: diethyl ether (1:9), 4 ml, was added and the mixture again shaken for 30 sec. The samples were centrifuged in a Damon IEC Division CRU-500 centrifuge at 260 g for 10 min. at room temperature to solidly pack the precipitated protein. The supernatant was decanted into 50 ml graduated plastic conical centrifuge tubes (Nalgene Company), with caps. This procedure was repeated with a second 4 ml

quantity of formic acid: ether mixture and the supernatant combined with the first. As a routine check for extraction efficiency, at this stage, the precipitate was irradiated under a u.v. light source. If no porphyrin fluorescence was visible, the extraction was considered complete. 2 ml of 1.5 M HCl was then added to each supernatant; the tubes stoppered and vigorously shaken for 30 sec. Porphyrin is extracted into the lower HCl layer. Using a long-stemmed glass Pasteur pipette the lower layer was carefully transferred to small tinted glass vials and stored in the dark. All stages of the procedure are carried out under subdued light to reduce porphyrin degradation. The absorbance of the solution was read at the wavelengths 380, 408 and 430 nm in a 4 mm pathlength cell in a Unicam/Pye SP500 spectrophotometer.

Calculation:-

$$\frac{2 A_{408} - (A_{380} + A_{430}) \times 1.28 \times \text{ml HCl solution}}{\text{quantity of tissue (g)}} \\ = \mu\text{g protoporphyrin-IX/gram tissue}$$

3.4.4 Whole blood

Reagents: acetone 1 vol: to ethyl acetate 9 vol.

formic acid (98%) 1 vol: to diethyl ether 9 vol.

1.5 M HCl

0.156 M NaCl

Heparin

An adaptation of the method by Heller et al. (1971) used to clinically screen blood for erythrocyte porphyrin was used to estimate protoporphyrin-IX in whole chicken blood.

5 ml of blood was withdrawn from the wing vein into a heparinised syringe. A capillary aliquot was taken and the micro-haematocrit measured with a Hawksley micro Haematocrit reader (Gelman Hawksley Ltd, Surrey). Typically, at this stage, the sample was either assayed immediately or frozen at -70°C and stored until required. Duplicate 2 ml blood samples were pipetted into 15 ml plastic centrifuge tubes (Nalgene Company), with caps and 2 ml of acetone: ethyl acetate added. The mixtures were vigorously stirred for 1 min. with a glass rod. This treatment has the effect of dehydrating the red blood cells and permits the precipitate to pack solidly to the bottom of the tube. Four ml of the extraction mixture, formic acid: diethyl ether, was added and the mixture again stirred vigorously for 1 min. with a glass rod. The tubes were spun in a Damon/IEC Division CRU-500 centrifuge at 260 g for 10 min. to pack the precipitate.

The supernatant fluid was decanted into a plastic graduated centrifuge tube and extracted with a second 4 ml volume of formic acid: diethyl ether and the procedure repeated as before. Both supernatants were then combined. 2 ml of 1.5 M HCl was added to the supernatants, the tube stoppered and shaken vigorously for 30 sec. The volume of the lower HCl layer was recorded and transferred to a small tube using a long-type Pasteur pipette. However, at this stage it was found that small amounts of organic matter remained in the HCl. This was most effectively removed by placing the sample into a spin thimble, (Reeve Angel and Co. Inc.), packed with a small plug of Whatman GF/A filter support. The sample was eluted into a plastic 15 ml centrifuge tube by spinning at 400 g for 3 min. The absorbance of the solution was read, versus a blank of 1.5 M HCl at the wavelengths 380, 408 and 430 nm in a Unicam/Pye SP500 spectrophotometer.

Calculation:-

$$\frac{2 A_{408} - (A_{380} + A_{430}) \times 1.28 \times \text{ml HCl} \times 100}{\text{Haematocrit} \times \text{ml whole blood}}$$

= μg protoporphyrin-IX/100 ml blood

3.5 AMINO ACID ESTIMATIONS

Solutions

Methyl cellosolve (2-methoxy ethanol). A standard preparation purified for amino acid measurement.

Diluent solution. Equal volumes of ethanol and glass redistilled water.

4N Sodium acetate buffer (pH 5.5). The buffer was made up by adding 544.36 g to 1 litre of warmed distilled water. Sufficient glacial acetic acid was added to correct the pH to 5.51 ± 0.03 .

Ninhydrin reagent

10 g ninhydrin

1.5 g hydrindantin

375 ml of 2-methoxy ethanol were used to dissolve the above reagents while bubbling with nitrogen.

12.5 ml of sodium acetate buffer (pH 5.5) were then added.

The solution was immediately stored in a dark glass bottle.

Nitrogen was bubbled through before use.

Glycine standards

A stock solution was made by dissolving 20 mg of glycine in 1 litre of buffer giving a concentration of 20 $\mu\text{l/ml}$.

From this dilutions were made to another four standards of 15, 10, 5 and 2 $\mu\text{g/ml}$ concentration.

Method

100 μl of cuticle hydrolysate was made up to 4 ml with diluent. Duplicate samples of 1 ml were taken and added to 1 ml ninhydrin reagent and placed into a boiling water bath for 15 min. The standards were similarly treated along with 1 ml of diluent for a blank. After 15 min. the tubes were removed, allowed to cool and 5 mls of diluent added to each. The tubes were shaken together for 30 sec. and allowed to cool. The absorbances of the standards followed by the tests were measured on a Unicam SP500 spectrophotometer at 570 nm against a blank of diluent solution. The standard glycine curve was used to estimate the unknown samples.

Glycine recoveries

- A. 100 μl of cuticle hydrolysate was made up to 4 ml with diluent. 1 ml was taken and added to 1 ml of ninhydrin reagent. The sample was treated as before.
- B. 100 μl of cuticle hydrolysate was made up to 2 ml with diluent and 2 mls of the 20 $\mu\text{g/ml}$ glycine standard added. 1 ml of this solution was taken and added to 1 ml of ninhydrin reagent and treated as usual.

Table 3.1. An example of amino acid recoveries

Standard solution $\mu\text{g/ml}$ glycine	Absorbance	
2	0.000	Theoretical values: B-A = 10 $\mu\text{g/ml}$ glycine C-A = 5 $\mu\text{g/ml}$ glycine
5	0.119	
10	0.345	
15	0.533	
20	0.745	
unknowns		
A = 9.7	0.317	
B = 20.2	0.755	
C = 14.4	0.511	
Recoveries:	B-A = 105%	
	C-A = 97%	

C. 100 μ l of cuticle hydrolysate was made up to 2 ml with diluent and 2 mls of the 10 μ g/ml glycine standard added. As before 1 ml was taken and mixed with 1 ml ninhydrin reagent and treated as usual. Recoveries were carried out in triplicate after every new assay. The solutions were read, as before, at the wavelength, against a blank of diluent. Table 3.1 shows the typical recoveries obtained.

3.6 PREPARATION OF CONTROL CUTICLE HOMOGENATE

During this investigation, standard volumes of cuticle extract were used as controls with all measurements of cuticular mass and protoporphyrin-IX.

Preparation: whole eggs (n=10) were submerged together in EDTA solution for 2.5 h. The cuticles were removed from the egg shells by the method described in Section 3.2, and collected in a flask. The sample was made up to 100 ml with deionised water and homogenised using a Polytron, type PT 10 0D (The Northern Media Supply Ltd), homogeniser at full speed for 30 sec. 10 X 10 ml aliquots were dispensed into vials and stored in the dark until required.

Protoporphyrin-IX and amino acid were estimated in random samples of 1 ml, and run in duplicate with each assay.

3.7 NICARBAZIN TREATMENTS

Varying quantities of the coccidiostatic drug Nicarbazin (nicrazin) 98% purity, Merck Sharp and Domhe, Agvet, were dispersed into 100 mg capacity gelatine capsules. As a marker, to ensure identification of eggs from treated hens, 2 mg of the dye Sudan Black

were incorporated into each capsule along with each dose of drug. Sudan Black stains yolk lipid, and when the egg was opened it confirmed a treated individual. To each group (n=5 hens per group) a quantity of the drug was administered every 24 h, which normally followed oviposition. The capsule was placed in the birds' mouth with a small quantity of feed and gently directed towards the back of the throat to ensure its consumption. Control hens were administered an empty gelatine capsule in a similar fashion, every 24 h. The eggs laid following treatment, were weighed, and a broad-pole reflectance measurement recorded. Both control and treatment groups of eggs were assayed for total shell porphyrin i.e. membranes, true shell and cuticle (see Sections 3.4.1 and 3.4.2) and cuticle amino acid.

Tissue sampling

Prior to the administration of the drug, 5 ml of whole blood were withdrawn from the wing vein from all birds within each treatment group. The haematocrit was immediately measured and the samples stored at -70°C until required. After four days of nicarbazin treatment, blood sampling was repeated, as above, for all groups, and the hens killed by an intravenous overdose of sodium pentobarbitone. The shell gland pouches were removed from each bird, washed in isotonic saline (0°C) and stored at -70°C . Protoporphyrin-IX was assayed in both the shell gland tissue samples and the whole blood samples as described previously in Sections 3.4.3 and 3.4.4 respectively.

3.8 CHEMICALS

Acetone, AnalaR, BDH Chemicals Ltd, Poole, Dorset, England

Calcium chloride hexahydrate, BDH Chemicals Ltd

Diethyl ether, AnalaR, BDH Chemicals Ltd

Edicol Supra Pea Green H Stain, ICI Manchester, England

Ethyl acetate, AnalaR, BDH Chemicals Ltd

Ethylenediaminetetra-acetic acid (EDTA) disodium salt, AnalaR, BDH Chemicals Ltd

EDTA tetrasodium salt, Prolabo, 12, rue Pelee, F7591, Paris

Formic acid (90%) AnalaR, BDH Chemicals Ltd

Glacial acetic acid, AnalaR, BDH Chemicals Ltd

Hydrindantin, Sigma Chemical Co., Poole, Dorset, England

Hydrochloric acid, constant boiling (20.24%), BDH Chemicals Ltd

Methyl cellosolve (2-methoxy ethanol)

Nicarbazin (nicrazin), batch No. ABJ 1783, 98% purity, Merck

Sharp and Dohme, Agvet Ltd, Hodesdon, Hertfordshire, England

Ninhydrin reagent, Sigma Chemical Co.,

Protoporphyrin-IX dimethyl ester, Sigma Chemical Co.

Saline solution (0.9%)

Sodium acetate, BDH Chemicals Ltd

Sodium pentobarbitone, Expiral, Ceva, Southampton, England.

EXPERIMENTAL WORK

4.0 EXPERIMENTAL WORK

4.1 PHYSICAL STUDIES ON EGG SHELL COLOUR: THE EFFECT OF AGE, EGG SIZE AND EXPOSURE TO SUNLIGHT ON THE SHELL COLOUR OF DOMESTIC FOWL EGGS

4.1.1 Introduction

It has been demonstrated by many research workers that, as the commercial laying hen ages, not only does egg production fall, but egg shell quality also deteriorates (e.g. Perek and Snapir, 1970; Hamilton et al., 1979; Roland, 1976 and Nys, 1986). In many cases these changes can be reversed by causing egg production to be paused by means of forced moulting.

One aspect of shell quality that is of major importance to the egg industry, namely the decrease in shell colour that takes place as the hen ages, has received comparatively little attention. Such research as has been carried out on this subject has identified that the problem exists, but has involved experiments using only small numbers of birds with eggs sampled at large intervals over short periods of time. Assessment of egg colour in these studies was mainly by subjective means (Hall, 1944; Hunton, 1962; and an ADAS report, Warner, 1977, unpublished).

There is a need for a more systematic objective study of the physical factors affecting colour loss. In the first instance an investigation is required on the extent to which deterioration occurs and its variability within a flock. More information is also needed on the relationship between egg size and shell colour and on the observation by Blow et al, (1950) that light causes fading to occur in pigmented shells.

4.1.2 Aims

The experiments described in this section had the following objectives:-

To assess the day to day variability in the colour of eggs from individual hens over a short period of lay.

To monitor changes in the intensity of shell colour using reflectometry, as the laying cycle progresses.

To investigate the relationship between egg size and shell colour.

To examine the effect of exposure to sunlight on shell colour.

4.1.3 Experimental procedures

1. A short term study of individual hens.

A series of eggs (n=26) were collected daily over a period of around 30 days from two hens, to investigate the variability of colour. The eggs were weighed and a broad-pole reflectance measurement recorded immediately after lay. Appendix 1 reviews the background and development of egg shell reflectometry and describes the procedure of measuring colour, throughout the present investigation in greater detail. The final section of the Appendix presents the results of a test on the stability of the reflectometer used to assess colour intensity throughout this study.

2. The effect of age on shell colour in a small flock.

A flock of ninety one ISA Brown Hybrid hens (aged 25 weeks) were individually numbered and housed in cages. Eggs were recorded daily. For a period of 50 weeks, i.e. from 25 to 75 weeks of age, one egg was collected from each member of the flock every fourteen days.

The eggs were pencil marked around the equator and washed to remove adhering dirt, before recording weight to the nearest 0.1 g. A broad-pole reflectance measurement was taken, normally within 30 min of oviposition. If the eggs were left for any period of time before recording reflectance, they were stored at 4°C, in the dark.

For an extended period of twenty weeks lay to 95 weeks of age, reflectance was continued to be recorded from 18 members of the flock at fourteen day intervals as before. Limited housing space, unfortunately, prevented all members of the flock from being kept for the extended study.

3. The effect of egg size on shell colour.

At 26 weeks of age, 58 eggs were taken at random from different birds within the test flock. The eggs were weighed as before and a reflectance measurement recorded from both the broad and narrow poles. The effect of egg size on the intensity of colour was compared using linear regression analysis.

Calculation of egg shell surface area.

A number of methods are available (see Hughes, 1984), to estimate surface area. However, many require length and breadth measurements. An algorithm which compares well with other methods (Hughes, 1984) has been put forward by Carter (1974) and requires only a weight measurement. Shell surface area in cm^2 was measured using the algorithm $3.9782 W^{0.7056}$, where W = fresh egg weight in grams. The coefficients of the algorithm were mathematically derived from data obtained previously by Carter (1974) from length, breadth and weight measurements from a series of eggs.

4. The effect of sunlight on shell colour

A quantity of eggs ($n=28$) were randomly taken from different birds from within the test flock. Reflectance measurements were immediately recorded from the broad-poles. The eggs were divided into two groups. One group was placed in a darkened cupboard for use as controls. The remainder were left exposed to sunlight (July mean day length: 16.5 h; hours of bright sunlight: 4.2 h, The Meteorological Office Monthly Weather Report, 1987) in the laboratory for three weeks. Reflectance measurements were repeated on both sets of eggs.

4.1.4 Results

1. Individuals

The study of individual hens within the test flock has shown that shell colour, when measured daily, over short periods (around 30 days) fluctuates over a range of up to ± 7.25 reflectance units. This variation was shown to be normally distributed when analysed by analysis of variance (ANOVA) of standard deviations from the mean. Deviation from the mean reflectance was relatively small when egg shell colour was measured from 2 hens, as shown in Figure 4.1 and the reflectance means \pm SDEV from the hens were as follows, viz; 32.5 ± 2.17 and 41.4 ± 1.43 . When the change in reflectance from the first egg was regressed with age, no obvious correlation was observed, although the overall trend was to slightly lose shell colour. Examples of the data from hens sampled at two weekly intervals from week 25 to 95 are shown in Figure 4.2. Most hens over this period show a definite trend, with reflectance rising with time. Some individuals, however, demonstrated the ability to produce eggs with persistent shell colour, whereas other lose colour quickly and others produce eggs with greater colour intensity with age.

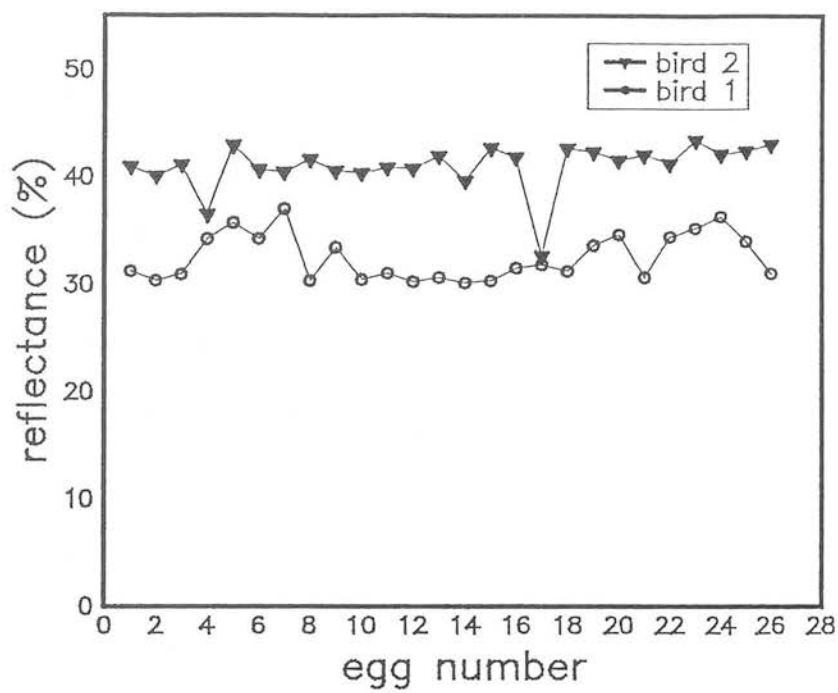


Figure 4.1 Reflectance values of eggs laid in succession from two individual hens over a short period of 26 d, early in the laying cycle.

Figure 4.2 Reflectance values of eggs (sampled every 14 d) from six individual hens over 70 weeks of lay.

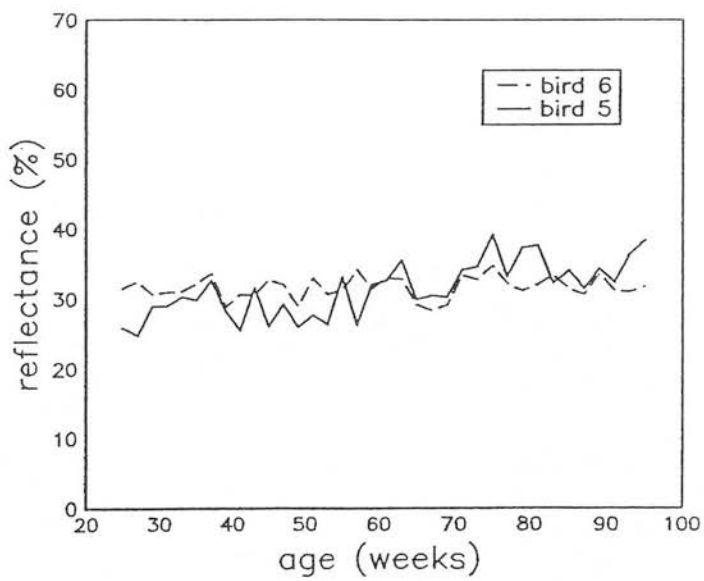
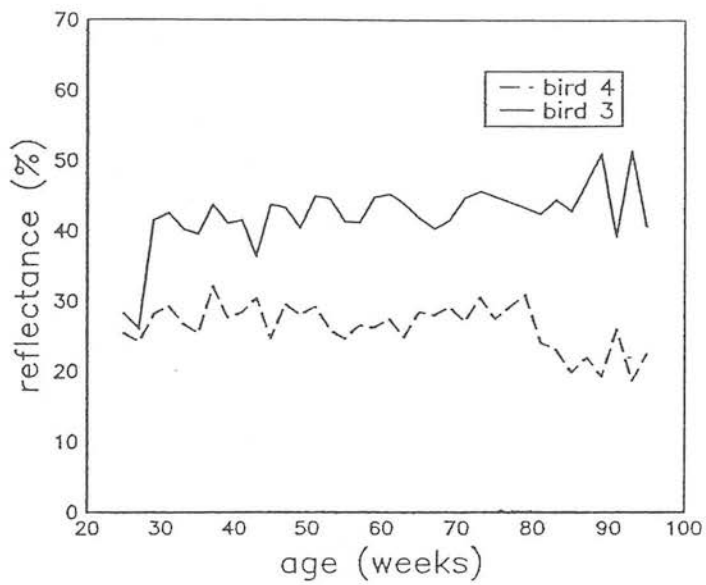
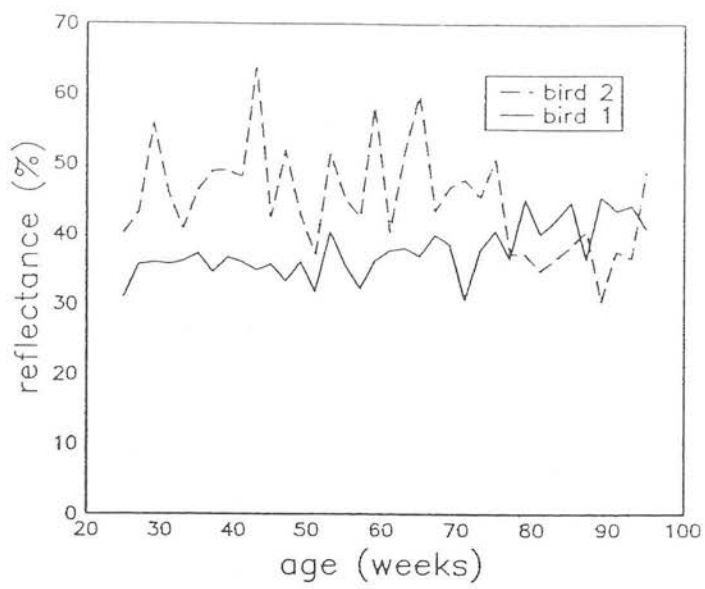


Table 4.1

Variability of the flock's shell colour with age

Age (weeks)	25	35	45	55	65	75	85	95
Variability (%)	15.4	16.8	16.1	17.6	17.8	19.0	19.5	20.0

n=91 eggs per sample

2. Flock

Shell colour from each sample of eggs in the laying cycle was extremely variable and a wide range in colour intensity was observed. Table 4.1 shows the variability in colour at selected stages in the laying cycle. The mean flock (n=91) shell reflectance at week 25 was 31.47 ± 0.56 SEM. During the following two weeks, shell colour was reduced, but from this point, colour fluctuated with age but the overall trend was to lose shell colour. By week 50 there had been a loss of shell colour which corresponded to a reflectance change of 6.43 units. A one-way ANOVA showed that the mean change in reflectance values at each two weekly point, with age, was significant, $F=2.08$ ($P<0.05$). Plate 2 shows the typical range in shell colour from the ISA Brown flock, as exemplified by eggs taken from different hens at week 30.

Loss of shell colour over the period was more accurately determined by calculating the change in reflectance at each sampling point when compared to week 25. Analysis of the changes using a Student t-test demonstrated that a change of 0.6 units from the mean value at week 25, was significant ($P<0.5$), and a change of 2.01 units was highly significant ($P<0.05$).

Linear and quadratic regression models were fitted to both sets of data. The changes with age (from week 25-75) most closely fitted a quadratic regression line as shown in Figure 4.3 by ANOVA using the least squares method (Steel and Torrie, 1980); $Y=0.058 + 0.213X - 0.00173X^2$ ($P<0.05$) where Y =change in reflectance and X =age (weeks). When a proportion (n=18) of the hens were sampled over the extended period of 20 weeks, up to week 95, the data continued to fit a

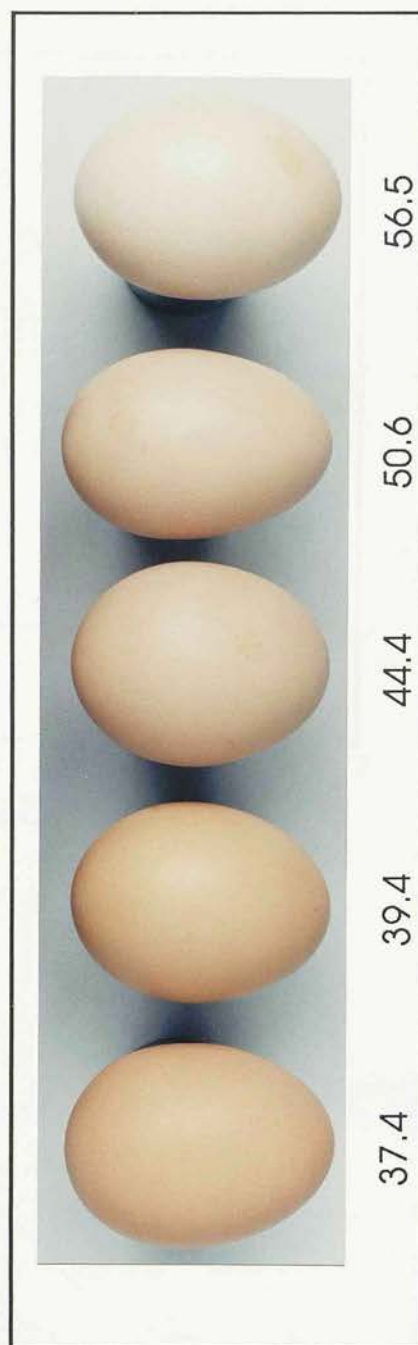
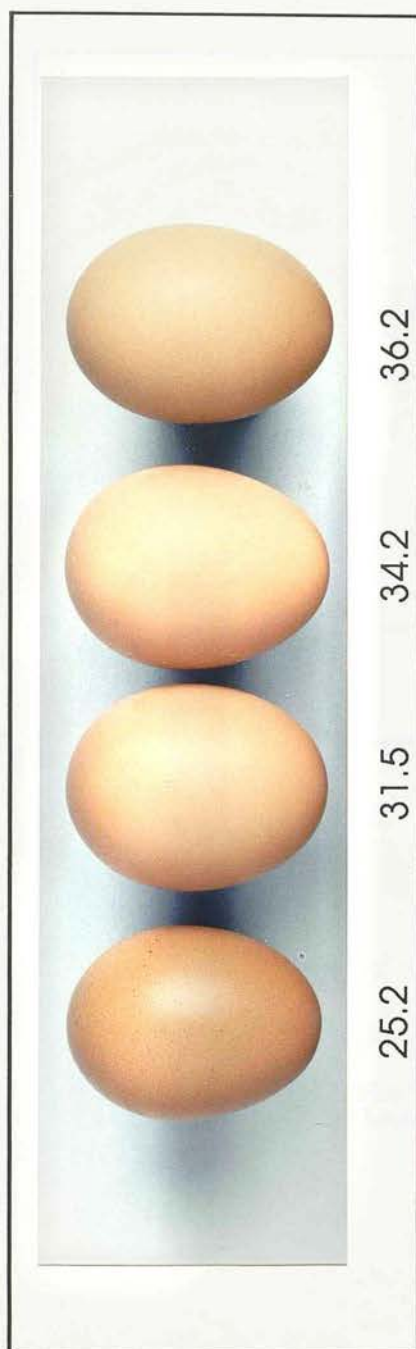


PLATE 2

The typical range in shell colour intensity from the test flock (ISA Brown Hybrid) as exemplified by eggs from 9 hens at week 30. The figures represent reflectance measurements recorded from the broad poles.

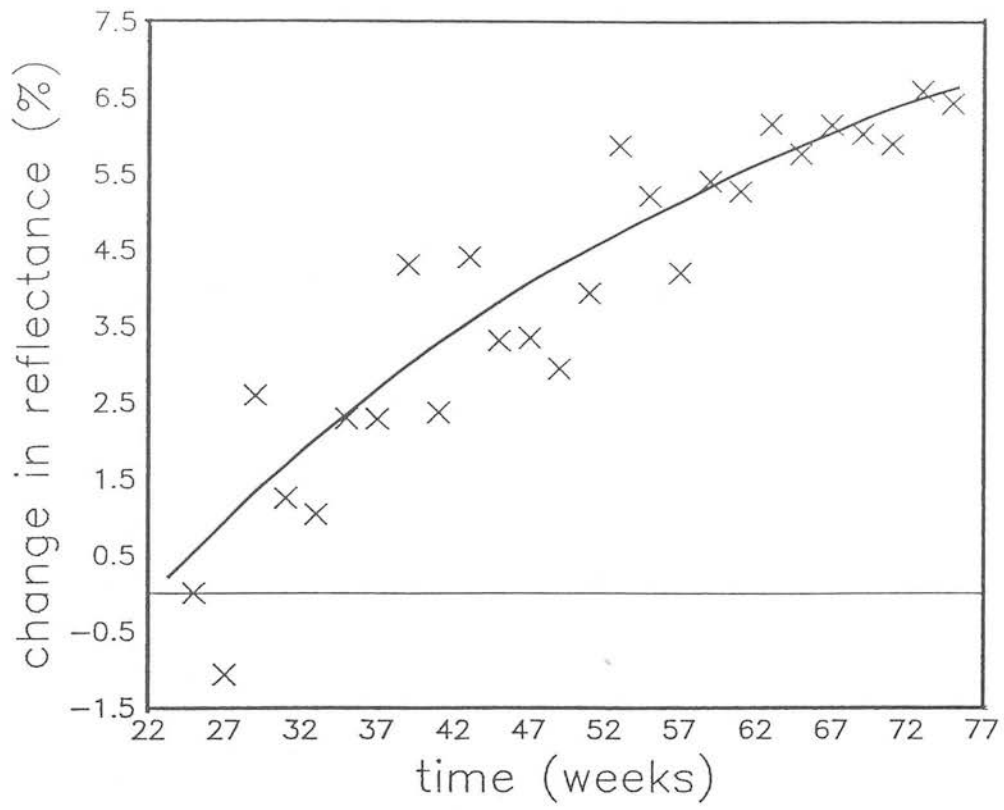


Figure 4.3 The effect of age on the change in shell colour intensity from a small flock of hens (n=91), between 25 and 75 weeks in the laying cycle.

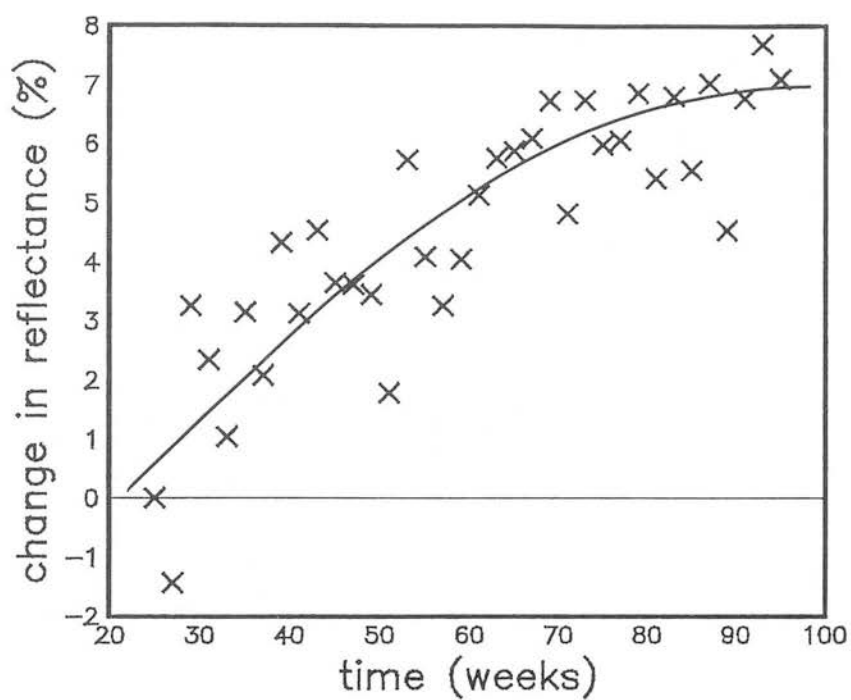


Figure 4.4 The effect of age on the change in shell colour intensity in a proportion of hens (n=18), taken from the test flock, to an extended point in the laying cycle of 95 weeks.

Table 4.2

The effect of exposure to sunlight on shell
reflectance, in comparison to eggs stored
in darkness

Group	Reflectance (broad-pole)	
	Measurement 1	Measurement 2
Controls ¹	37.15+1.91	37.70+1.97
Exposed ²	35.30+1.90	51.79+1.90**

means + SEM based on 14 eggs

1 eggs stored in darkness for 21 days

2 eggs exposed to sunlight for 21 days

** P<0.01

quadratic regression model, (see Figure 4.4); $Y=0.66 + 0.163 X-0.011 X^2$ ($P<0.05$) where Y = change in reflectance and X =age (weeks). The loss of shell colour began to slow down at around 63 weeks of age, reducing rapidly by week 93.

3. Egg shell colour, size and age

From a random sample of 58 eggs from different birds at week 26, there was no relationship ($P>0.05$) found between shell colour (Y), measured at either pole, or a mean of the two values, and that of egg shell surface area (X). Sampling was repeated at later dates, viz; 35 and 50 weeks of age, but again no relationship was found between shell colour and surface area.

4. The effect of light on shell colour

When eggs ($n=14$) were left exposed to sunlight in the laboratory for 3 weeks and their colour intensity compared with control eggs ($n=14$) which were stored in darkness there was a significant ($P<0.01$) mean loss of colour intensity of 16.5 reflectance units, corresponding to a reduction in colour of 47% (see Table 4.2). Although the control eggs reflectances varied slightly in shell colour at the second reading, it was not significant ($P>0.05$). The differences are probably due to small variations of the point on the egg shell surface at which the second readings were taken.

4.1.5 Discussion

The results show that as a flock of commercial laying hens (ISA Brown Hybrid) age there is a corresponding loss of shell colour, when measured by reflectometry. The situation deteriorates with increasing age, and is in keeping with the early observations of Hall (1944),

Hunton (1962) and an ADAS report (Warner, 1977, unpublished) and those of a more casual nature by the poultry industry. Variability of shell colour between individuals within the flock was extremely large. Each member of the flock also produced eggs of variable colour intensity when reflectance was recorded daily (see Figure 4.1). However, in terms of visual perception of colour the fluctuation is small and most birds were capable of maintaining persistent shell colour over short periods. At this level of study (i.e. up to 26 days) no significant correlation of change in shell colour from day one, was found although reflectance increased slightly. The variation observed more closely highlights the sensitivity of the reflectometer used to monitor colour throughout the study.

The long term study of the flock, between 25 and 75 weeks of age showed shell colour to be very variable, ranging from 15-20% (and increasing with age) at each two weekly sampling point. The spread of shell colour with eggs laid by individuals was large with each sample (n=91) containing high proportions of pale coloured shells. Individuals over this period produced eggs with reflectances peaking and troughing, but in most cases the trend was to lose shell colour intensity with increasing age. Eggs from some birds, however, lose colour more quickly than others. A small number within a flock will produce eggs which are stable in colour intensity throughout the laying period. Other hens produced eggs with increasing colour intensity with time, and generally were less variable in shell colour.

The results of the changes in flock reflectance with age, between week 25 and 65, was 0.6 units per month, but this loss began to slow down at around 73 weeks of age. Over the extended study period



colour loss was shown to dramatically slow down, eventually reaching a point at around 83 weeks where the change in the loss of colour was negligible and failed to be significant. The maximum loss of shell colour with the two groups, $n=91$ and $n=18$ was 6.6 and 7.7 units respectively which indicated that the number of the hens studied in the large flock could have been reduced to observe a similar effect. The loss of shell colour over the study was relatively small in terms of visual perception of colour and the reduction was less dramatic than expected. Egg size appears to have no effect on the colour of an egg, when it was measured at specific points (26, 35 and 50 weeks) in the laying life of the hen. The increasing change in egg size and reflectance were closely correlated at specific ages. However, age is the factor which contributes to the majority of this relationship observed. Large eggs have been shown to be as intensely coloured as small eggs at selected stages in the laying period and it appears that the individual's capability to produce egg shell pigment which is the important factor rather than egg size.

The results have also shown that the pigment associated with egg shells, when exposed to direct sunlight causes shell colour to fade markedly within a few weeks. However, this aspect is controllable if the eggs are stored in darkness prior to marketing. Colouration in wild species egg shells offers camouflage protection (see Plate 1 and Section 1.3.2). This is especially true of eggs left unattended for long periods during incubation (Westmoreland and Best, 1986). Shell colour intensity is therefore important to crypsis of the egg, protecting the developing embryo from predators. It appears from the results presented here that it may also be disadvantageous to leave

eggs exposed to strong light (including the problem of overheating) because the shell colour may be reduced and therefore lower the protection afforded by crypsis. Conversely, loss of colour intensity may enhance cryptic protection with changes in the surrounding nest-site during the incubation period.

From a marketing point of view it is probably persistency of shell colour which is important to the producer throughout a flock's laying period. The results have shown that in one commercial strain of hen, acceptable shell colour quality may be maintained up to 80 weeks of age. However, down-grading may result from increased abnormalities of shell. Breeders looking for persistency of colour would benefit from monitoring individuals over at least 70 weeks of lay to identify birds within a strain meeting this requirement. These individuals may be used in selection for the long-term production of dark-brown egg shells.

Reflectometry has been shown to present a method which offers a high degree of accuracy when used to monitor shell colour over considerable periods of time.

4.2 PIGMENT LOCALISATION WITHIN THE DOMESTIC FOWL'S EGG SHELL

4.2.1 Introduction

An early study by Tamura and Fujii (1967a) indicated that the pigments present in the domestic fowl egg shell were distributed evenly throughout the shell. In Japanese Quail Coturnix coturnix japonica, the cuticle was shown to contain the majority of the shell pigment. Pigmentation occurring throughout the palisade layer was thought to form the ground colour of the egg (Tyler, 1966, 1969). Later Lucotte,

et al. (1975) demonstrated that the ground colour of many cryptically coloured egg shells (such as the Japanese quail) was associated with the true shell. A study of wild species egg shells, by Baird et al. (1975) showed that many pigments which are sometimes thickly deposited, are confined to the outermost layer of the egg shell. However, the majority of pigment in the egg shell of the domestic fowl on close inspection appears evenly distributed with only occasional superficial speckling. It is important, therefore before investigating shell colour in more detail that the precise location of the pigments within the shell responsible for expressing colour is identified.

4.2.2 Aims

An investigation was designed to clarify, in the first instance, the locality of the egg shell pigment which is responsible for colouration of the hen's egg shell.

4.2.3 Experimental procedures

A sample of eggs (n=47) was collected at random, from different birds within the ISA Brown flock at week 26 of lay. The eggs were individually marked, weighed and a reflectance measurement recorded from each pole of the eggs. The eggs were soaked in EDTA (0.156 M) solution for 2.5 h and the cuticles washed from the shells as previously described in Section 3.2. After allowing the eggs to dry, the reflectances of the eggs (now devoid of cuticle) were recorded at each pole. As a comparison the reflectances of 20 white eggs from a commercial laying strain (Ross white) were also recorded. The results were compared using a Student's t-test and linear correlation analysis of the variables.

4.2.4 Results

Table 4.3

The effect of cuticle removal on egg shell reflectance

Reflectance	Condition of egg shell		White egg shell ²
	Normal ¹	Devoid of cuticle ¹	
Broad-pole	36.21+0.98	64.19+0.77**	82.9+0.59
Narrow-pole	40.26+1.12	53.02+0.96**	83.4+0.66

1 mean + SEM n=47 eggs

2 mean + SEM n=20 eggs

** P<0.01

Table 4.4

Egg shell correlation coefficients of normal and devoid of
cuticle egg reflectances + 95% confidence limit

<u>REFLECTANCE</u>	Normal egg Narrow-pole	Devoid of cuticle Narrow-pole
Normal egg Broad-pole	0.8108+0.015**	NS
Devoid of cuticle Broad-pole	NS	NS

n=47 eggs

** (P<0.01)

NS no significant relationship

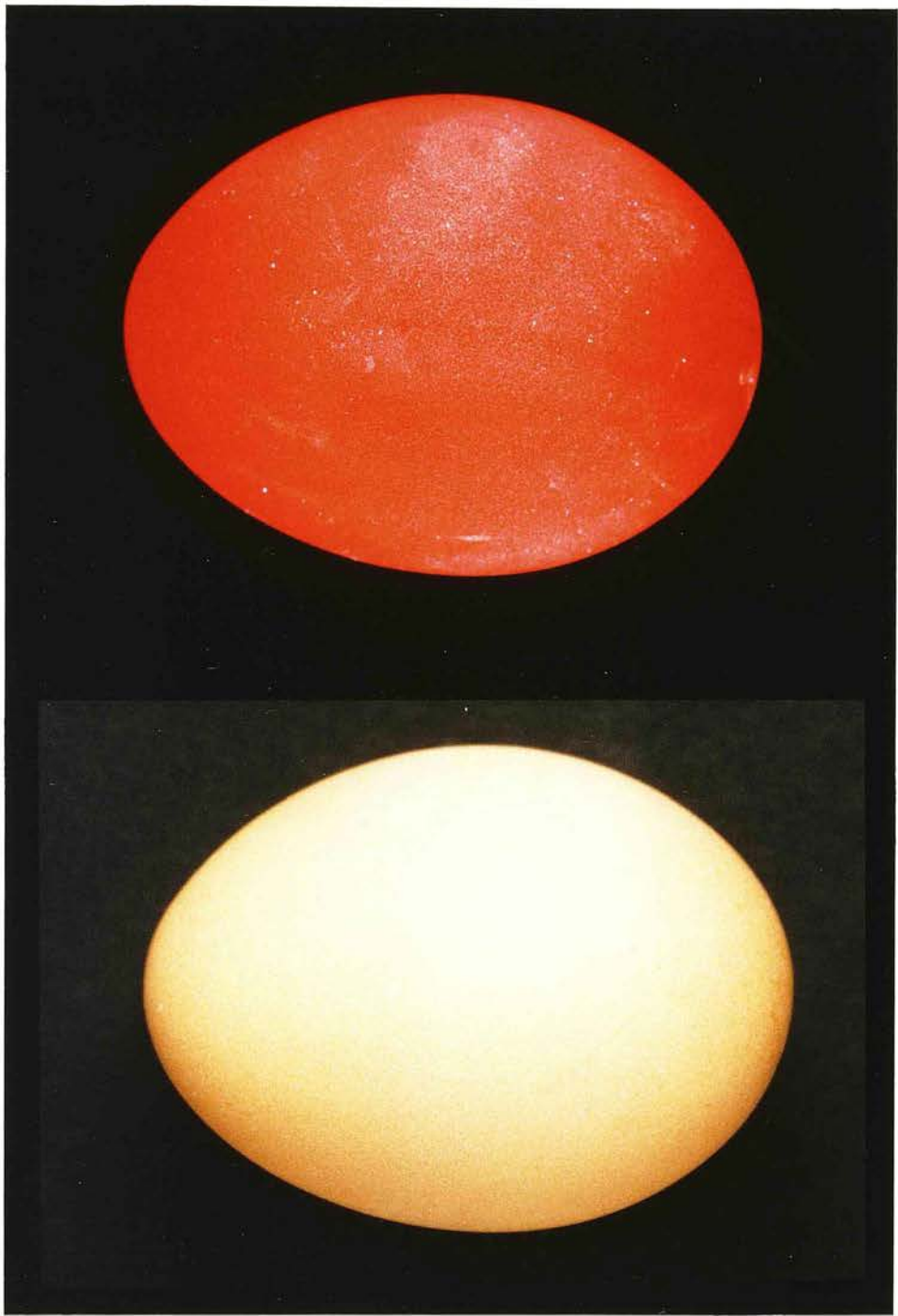


PLATE 3

The domestic fowl egg viewed under normal light (bottom) and irradiated under u.v. light (top). The intense orange-red fluorescence from the same egg (top) indicates the presence of porphyrin within the cuticle.

The results presented in Table 4.3 demonstrate that when normal brown eggs are devoid of cuticle, shell reflectance when measured at the broad and narrow poles significantly ($P < 0.01$) rises by 28.0 and 12.8 units respectively. This rise in reflectance constitutes a loss in shell colour of 43.6% and 24%. Subsequently, reflectance of normal white egg shells were significantly higher than those from brown shells. Cuticle-less brown egg shells had a lower reflectance than normal white shelled eggs. A significant relationship (see Table 4.4) was found between normal broad and narrow-pole reflectance in the sample. However, the relationship was not found when the eggs were devoid of their cuticles. When cuticle-less eggs were irradiated under a u.v. light, the intensity of fluorescence was markedly reduced when compared with that from normal eggs, with intact cuticles. Similarly, cuticle samples suspended in distilled water also fluoresced with greater intensity than cuticle-less shells. Plate 3 shows the fluorescence of an egg irradiated under a u.v. light and the same egg viewed under normal light.

4.2.5 Discussion

The results have shown that when the cuticle from eggs is removed, there is an increase in reflectance recorded from the underlying true shell portion. The increase in reflectance demonstrated a dramatic loss of shell colour. Thus, the cuticle acts as a carrier of pigment and the colour of eggs from domestic fowl is due to this coloured coating. Nevertheless, the lower reflectance of true shell, when compared with white egg shells, indicates that some pigment is left within the true shell when the colour which is

associated with the cuticle is removed. Speckling, present on some fowl egg shells was removed along with cuticle, which demonstrated their association with cuticle, which is in agreement with the observations of Baird et al. (1975) who studied wild bird's egg shells.

The broad pole of the normal fowl's egg was some 4.05 reflectance units lower than the narrow pole which demonstrates it is responsible for carrying more pigment than the narrow pole. No relationship was found between normal and devoid of cuticle broad pole reflectance which indicates that cuticle colouration is not dependent upon the underlying shells colour.

Past workers have suggested that the darker broad pole and high incidence of speckling, in comparison with the narrow pole may be a result of the broad end of the egg leaving the shell gland first at oviposition. It may therefore adsorb the majority of pigment present in the epithelial lining of the pouch. However, a study by Wood-Gush and Gilbert (1969) confirmed the work of Olsen and Byerly (1932) and showed that the orientation of the egg at oviposition is random and probably has no biological significance. The broad-pole may possess more cuticular pigment in the fowl's egg because it is in contact with a larger number of secretory epithelial cells as it rotates (Welty, 1962) within the shell gland. Speckling and mottling is probably a result of the egg remaining stationary, for periods, within the shell gland pouch, thus pigment secretions aggregating upon the cuticular layer.

4.3 THE DEVELOPMENT OF TECHNIQUES TO ESTIMATE EGG SHELL
PIGMENTATION. THE APPLICATION OF TECHNIQUES TO INVESTIGATE EGG
SHELL CUTICLE AND ITS ASSOCIATED PIGMENT

4.3.1 Introduction

The results presented in Section 4.2 confirm that the cuticle of the hen's egg shell acts as the carrier of egg shell pigment, which is predominantly protoporphyrin-IX (Wells, unpublished), and that it is responsible for the expression of shell colour. For this reason the cuticle was chosen as the portion of the egg shell, throughout this study, to utilise as an indicator of the degree of egg shell pigmentation. Consequently, methods to remove the cuticle from an individual egg shell were devised (see Section 3.2). Techniques to allow cuticular mass and porphyrin content to be estimated were required as existing methods were unsuitable. For example, the pigment present in crushed egg shell was studied by Polin (1957) using treatment with 3 M HCl prior to extraction with an ethyl acetate-acetic acid mixture. While Polin's method enabled the spectrum of porphyrin to be studied the isolated cuticle fluoresced under u.v. light indicating that some porphyrin still remained. A method by With (1973) also proved ineffective in removing cuticular porphyrin (the samples fluoresced under u.v. light) and presented the added problem of foaming when the 5% v/v H_2SO_4 in methanol mixture was added to the crushed egg shell. These methods were time-consuming, requiring 2 or 3 extractions. Consequently, a method was needed which could be readily used to routinely extract cuticle pigment from large-sized samples. The method devised enabled the cuticle protein to be hydrolysed, based on a method refluxing with constant boiling HCl and formic acid mixture

(Bailey, 1967) and estimation of cuticular mass based on amino acid content (see Section 3.5). This solution was also found to be an excellent extraction mixture for protoporphyrin-IX, as indicated by a recovery versus time trial. The method relies on an enclosed hydrolysis/extraction system which requires only one volume of the mixture and reduces loss which may be associated with tube to tube transfers. Egg shell matrix proroporphyrin-IX can be estimated using an adaptation of the above method using the HCl-formic acid mixture to extract, cold, the porphyrin in egg shell.

4.3.2 Aims

- A. To study the effect of heating (the hydrolysis procedure) on the stability of protoporphyrin-IX and the effect of an enclosed or open system on recoveries.

To test the linearity of amino acid and porphyrin content in samples of cuticle homogenate.

To establish the levels of recovery of cuticle amino acid using the ninhydrin method.

B. Additional aims

To determine the pigment and amino acid content of cuticles from a selection of eggs from the test flock. The relationship between the physical estimations used to study shell colour (such as reflectance and egg size) are compared with the newly established chemical methods (cuticle and shell pigment, and cuticle mass).

To investigate the viability of reflectance as an indicator of overall shell colour intensity.

To study the effect the degree of cuticular cover possess on influencing colour intensity.

4.3.3 Experimental procedures

A. Recovery tests

The effect of heat on protoporphyrin-IX recovery

50 μ g of protoporphyrin-IX dimethyl ester (Sigma Chemical Co.) in 5% HCl (46 μ l) was spotted on to Whatman GF/A (7.0 cm diam.) filters, X10. These were placed in Oak Ridge centrifuge tubes and 20 ml of the mixture HCl-HC00H (see Section 3.3) added to each tube. The tubes were gassed with N_2 for 3 mins, and after tightening they were placed in an oven preheated to 94°C and heated for 22 h. After 3 h the tubes were removed, shaken vigorously for 30 sec. and returned to the oven. The tubes were allowed to cool and then centrifuged at 250 g for 10 min. The solutions were filtered through Whatman No. 54 (7.0 cm diam.) and then the optical densities measured at 380, 408 and 430 nm, against a blank of the HCl-HC00H mixture treated similarly.

A second group of protoporphyrin-IX samples (X10) was treated as above but left in the dark at room temperature (18°C) for 22 h. The solutions were filtered and the optical densities measured as before. This test was repeated at a later date. The data was compared using Student's t-test.

Gravimetric test

To determine whether any loss was associated with loosening the caps of the tubes during hydrolysis, to avoid the build up of

internal pressure, two groups (n=8 per group) of tubes containing the acid mixture (20 ml) were weighed, then placed into the oven at 94°C for 22 h. One group had the caps loosened slightly and the other tightly sealed for the duration of the heating. After heating, the tubes were re-weighed.

Amino acid recoveries of egg shell cuticle (as estimated by glycine determination)

The ninhydrin method shown in Section 3.5.6 was routinely employed to estimate cuticle mass throughout the course of this investigation. Normally, following an amino acid measurement, the degree of recovery was estimated. Actual and theoretical values of recovery were compared as shown in Section 3.5.6.

A test for linearity of egg shell cuticle protoporphyrin-IX and amino acid estimations.

Routinely during the course of the investigations of egg shell cuticle composition the linearity of analysis was verified. Control samples of cuticle homogenate as prepared in Section 3.6 were dispensed, in duplicate volumes of 5, 10, 20, 30 and 40 mls on to GF/A filters (9.0 cm diam.) and left aside to dry in a darkened fume cabinet. The samples were hydrolysed as before in the HCl-HC00H (5%) mixture and protoporphyrin-IX and amino acid estimated in the samples. The data were subjected to linear regression analysis.

B. Amino acid and protoporphyrin-IX content of domestic fowl egg shell

A selection of eggs (n=47) was randomly collected from the test flock, at 26 weeks of age. The eggs were cleaned, weighed and a reflectance measurement recorded from each pole. The cuticles were individually separated from the egg shells by soaking in 0.156 M EDTA for 2.5 h as described previously. Each cuticle was hydrolysed in HCl-HCOOH (5%) mixture for 22 h at 94°C (see Section 3.3) and the protoporphyrin content estimated spectrophotometrically, as described earlier, at 380, 408 and 430 nm. Normally a 2 mm pathlength cell was used; the porphyrin in solution was very concentrated, and this technique avoids loss associated with diluting the extract. 100 μ l was taken from each hydrolysate extract, and amino acid level (as glycine content) estimated spectrophotometrically, using the ninhydrin method described previously.

Protoporphyrin-IX content of domestic fowl egg shell devoid of cuticle

From above, 29 egg shells devoid of cuticle, were saved and stored in the dark before use. Protoporphyrin-IX was estimated by the procedure using a cold extraction with the acid mixture as described in Section 3.4.2. The quantity of 20% HCl, added to each crushed egg shell to neutralise CaCO₃ (depending upon shell weight) was on average 18.9 ml. Protoporphyrin-IX was

estimated spectrophotometrically, normally using a 10 mm pathlength cell.

4.3.4 Results

Table 4.5

Gravimetric test on acid loss during
hydrolysis at 94°C

	<u>Condition of tube</u>	
	<u>Sealed</u>	<u>Loosened</u>
Weight loss(g)		
Mean	0.53	3.17***
SEM	0.027	0.140

n=8

*** P<0.01

ANOVA showed that there was a significant weight loss when the acid mixture was heated. However, weight loss was reduced when cuticle was hydrolysed in the enclosed system with the caps tightened throughout the procedure.

Table 4.6

The effect of heat on protoporphyrin-IX
recovery

recovery μg	Temperature	
	18° C	94° C
Mean	50.66	50.15
SEM	0.228	0.310

n=10

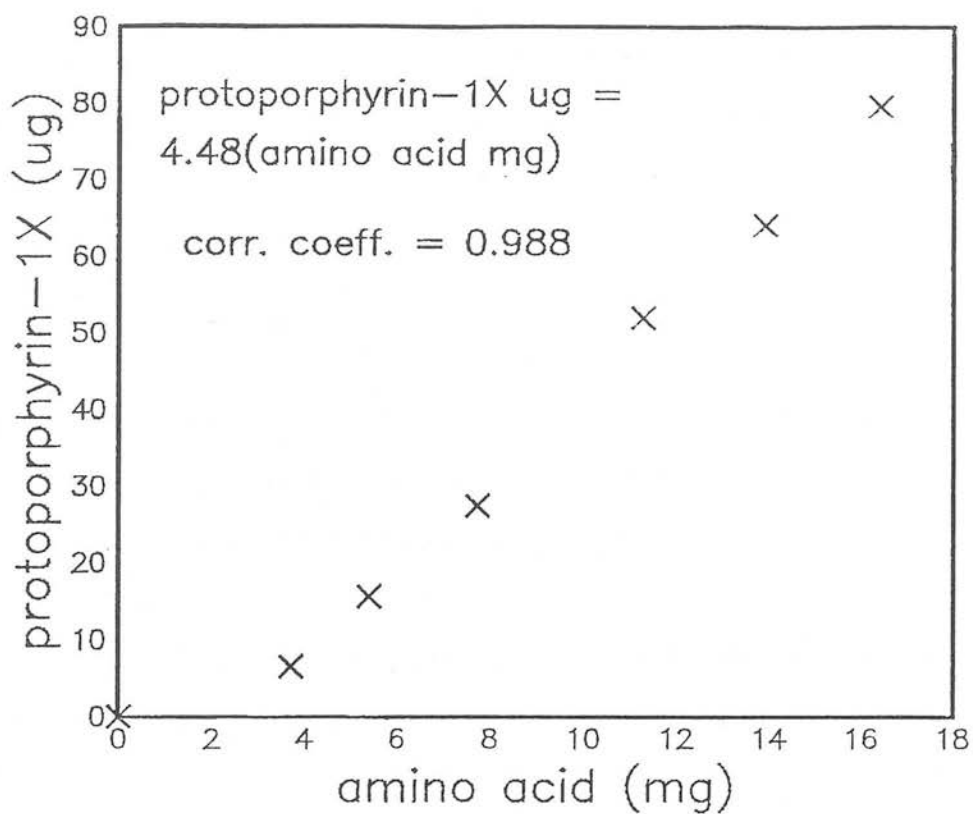


Figure 4.5 The linearity of amino acid and protoporphyrin-IX estimations in varying volumes (5 to 40 mls) of egg shell cuticle homogenate.

ANOVA showed that heat has no effect on the stability of protoporphyrin-IX in the 5% acid mixture and the recoveries from each treatment were not significantly different.

The preliminary investigation to compare the effect of heat on the volume of HCl-HCOOH (5%) mixture (by simulating hydrolysis conditions) within the Oak Ridge tubes with caps either tightly sealed or loosened throughout heating showed that there was a significant loss of acid mixture ($P < 0.01$, see Table 4.5) when the caps were loosened. This test indicated that there was the possibility of high protoporphyrin-IX and amino acid estimations due to concentration of the hydrolysate when the mixture evaporates. All hydrolysis of cuticle from this point was carried out under the enclosed system. The Teflon tubes were capable of withstanding damage when the build up of pressure occurred.

The result of the test on the effect of heat on the stability of protoporphyrin-IX (see Table 4.6) showed that heating for 22 h at 94°C has no significant effect on recovery of the porphyrin when compared with extractions at room temperature (18°C).

Estimations of protoporphyrin-IX and amino acid in varying quantities of cuticle homogenate demonstrated that a highly significant correlation coefficient \pm 95% confidence limit, ($r = 0.9989 \pm 0.018$, $P < 0.001$) exists between them. This indicates a high degree of precision with the techniques when used to estimate cuticle mass and its associated porphyrin pigment. Figure 4.5 shows the linear regression line with equation $Y = 4.48X$, where Y = homogenate protoporphyrin-IX and X = homogenate amino acid.

Table 4.7

Mean values of egg shell colour, protoporphyrin-IX and amino acid estimations

Egg shell estimation								
	whole egg		egg shell reflectance		Cuticle		egg shell	
	egg weight (g)	surface area (cm ²) ²	B.P ³	N.P ⁴	amino ⁵ acid (mg)	proto-IX ⁶ (μg)	weight (g)	proto-IX ⁶ (μg)
Mean ¹	65.54	75.97	36.90	39.45	13.94	60.69	5.17	82.98
SEM	0.83	0.68	1.05	0.99	0.43	5.85	0.12	3.78

1 means based on 47 eggs

2 surface area is estimated by an algorithm of Carter (1975)

3 broad-pole

4 narrow-pole

5 amino acid, as estimated by glycine content

6 protoporphyrin-IX

Table 4.8

Egg shell correlation coefficients and 95% confidence limits between estimates of reflectance, egg size, cuticle mass and its porphyrin content in a selection of different eggs from the ISA Brown flock

TRAIT	Shell weight	proto-IX ³ /true shell	proto-IX ³ /cuticle	cuticle mass	surface area	reflectance N.P. ²
reflectance B.P. ¹	-0.4904** +0.22	-0.4941** +0.22	-0.6314** +0.18	0.0419	0.1571	0.6644** +0.16
reflectance N.P. ²	-0.2866	-0.1279	-0.5845** +0.19	0.007	0.2796	
surface area	0.3813	0.4586* +0.23	0.2033	0.3925* +0.25		
cuticle mass	0.3394* +0.27	0.4217* +0.28	0.4480* +0.23			
proto-IX ³ /cuticle	0.4924** +0.22	0.6007** +0.19				
proto-IX ³ /true shell	0.6185** +0.18					

estimates based on 47 eggs

1 broad-pole

2 narrow-pole

3 protoporphyrin-IX

* P<0.05

** P<0.01

Amino acid recovery values from cuticle hydrolysates were high, with normal acceptable values greater than 95% (see Table 3.1).

The mean values and ANOVA of egg shell colour, protoporphyrin-IX and amino acid estimations are summarised in Table 4.7. The egg shell surface area calculations are based upon egg weight. Broad-pole reflectance, as demonstrated earlier was lower than that of the narrow-pole, by on average 2.6 reflectance units, $n=47$ eggs. Both measurements of reflectance were considerably variable from the sample, i.e. 19.5 and 17.2 per cent, respectively. The egg shells devoid of cuticle contained a significantly greater concentration of protoporphyrin-IX pigment, of $22.3 \mu\text{g}$, than the individual cuticles. Total shell porphyrin (true shell plus cuticle) was $144 \mu\text{g} \pm 25.9$ SDEV. Egg shell pigment content, similar to reflectance is very variable within the flock. A study by Schwartz et al. (1975) showed that Rhode Island Red egg shell (the whole shell crushed) contained around $300 \mu\text{g}$ of pigment. The sample size was small and no indication of the variability between eggs was given.

Cuticular mass, as estimated by glycine content was $13.9 \text{ mg} \pm 2.93$ SDEV, and is slightly lower than the mass of 18 mg reported by Cooke and Balch (1969) using a gravimetric estimation of a bulk sample of dried cuticle from an undefined breed of domestic hen.

The egg shell correlation coefficients between various measurements on eggs are presented in Table 4.8.

Physical and chemical correlations

Highly significant negative correlations ($P<0.01$) were found between estimates of shell colour (broad-pole reflectance) and those of egg shell weight, true shell and cuticle protoporphyrin content. There

was also, as would be expected, highly significant ($P < 0.01$) positive correlation between measurements of reflectance taken at each pole. No significant correlations were found between measurements of shell colour with either cuticle mass or egg shell surface area. However, correlations between surface area and porphyrin within the true shell or the cuticle were significant ($P < 0.05$). The porphyrin content of both cuticle and true shell correlated highly significantly ($P < 0.01$) with egg shell weight.

Chemical correlations

A significant positive correlation ($P < 0.05$) existed between cuticle mass and its porphyrin content. Further correlation between true shell and cuticle porphyrin contents was highly significant ($P < 0.01$).

4.3.5 Discussion

From the results on the development of techniques it is clear that estimations of egg shell cuticle mass (by amino acid estimation) and its associated porphyrin content show a similar degree of accuracy and are repeatable. The hydrolysis procedure has been shown to have no effect on the stability of protoporphyrin-IX within the acid mixture. Good recoveries of amino acid in cuticle hydrolysates were also obtained. Similarly the egg shell devoid of cuticle, was assayed for protoporphyrin-IX successfully using the cold extraction with the acid mixture. When left to stand overnight, the egg shell mineral matter completely dissolved in the acid mixture leaving only the shell membranes in solution. It was also later found that the cold

extraction procedure was suitable for estimating cuticle protoporphyrin-IX, as it left the cuticle translucent and free from porphyrin fluorescence when irradiated by a u.v. light source. This method was employed when estimation of cuticle mass was not required.

Again it has been demonstrated that the broad-pole is the darkest area of the egg shell. A significantly greater amount of protoporphyrin-IX pigment has been shown to be present in egg shell devoid of cuticle when compared with the cuticle alone. Egg shell devoid of cuticle has an average weight of 5.2 g with porphyrin (μg) content of 82.98 ± 20.37 SDEV. Cuticle mass averaged 13.9 mg with a porphyrin content of 60.60 ± 31.48 SDEV. The cuticular layer, therefore, contains $4354 \mu\text{g}$ protoporphyrin-IX/gram of cuticle and the egg shell only $16.1 \mu\text{g}$ protoporphyrin-IX/gram of shell. Weight for weight the cuticle contains over 300-fold more pigment than the true shell. Regardless of the low mass of the cuticle and an average thickness of only around $10 \mu\text{m}$ (Simons, 1971) it carries approximately 42% of the egg shell's total pigment. This study has again demonstrated the relative importance of the cuticle as a carrier of egg shell pigment and reinforces the physical studies of section 4.2 using reflectometry which highlighted pigment localisation within the egg shell.

The observed values for the correlation coefficients between egg shell colour (as measured by broad-pole reflectance) with shell weight, true shell pigment and cuticle pigment are negative and highly significant. A negative value results in these cases due to reflectance being inversely related to shell colour intensity. These relationships show that, as was expected, darker egg shells contain a

greater concentration of pigment, in both the true shell and cuticle, than lighter coloured eggs. Darker eggs also possessed more egg shell as was indicated by the high correlation between reflectance and shell weight. Positive correlations between shell colour and specific gravity have been demonstrated by Grover et al. (1980) and between shell colour and thickness (Godfrey and Jaap, 1949). A more recent study by Campo and Escudero (1984) found a positive relationship between shell colour (when measured subjectively by comparison with standards) and thickness. The results presented in the present study and those by other workers support the belief that dark-brown-shelled eggs contain more egg shell and may be stronger and thicker than lighter coloured eggs. These findings are of importance to breeders selecting for improvement in egg shell quality. If these traits can be selected for it may reduce the loss during egg handling which is between 5 and 7 per cent (Peterson, 1965), associated with damage in commercially produced eggs. The cuticular mass of egg shells also correlated significantly and positively with shell weight. This correlation indicates that eggs which have heavy shells possess more cuticle than eggs with lower shell weights. However, the contribution which the cuticle itself plays towards shell strength has not been fully demonstrated. By comparing the thickness of normal and egg shells devoid of cuticle it was shown by Belyavin and Boorman (1980) that the cuticle significantly contributes to shell thickness. However, the contribution the cuticle presents to shell strength is unclear, but as shell thickness is known to influence shell strength (Simons, 1971) the presence of an intact cuticular layer may maintain shell strength. The relationship between shell weight and cuticle mass

may be expected in view of both the calcium and protein (the cuticle) of the shell being secreted from the shell gland surface epithelial cells prior to oviposition of the egg (Richardson, 1935). The change from the deposition of the true shell to the deposition of cuticle may be expected to have a common relationship, i.e. when calcification ceases, cuticular deposition begins. Cuticular secretion may be dependent on the point in time when true shell secretion stops and upon the quantity laid down prior to cuticle deposition beginning. At present, it is not known whether the factors controlling the process of change are under nervous or hormonal control. The mass of cuticle on the egg shell was shown to be weakly related to its pigment content. True shell pigment in turn is also correlated with cuticle pigment which was not apparent from reflectance measurements of normal and eggs devoid of cuticle. Shell pigment therefore appears to be dispersed throughout its depth, in comparison to the superficially coloured, thin cuticular layer. Reflectance would therefore fail to represent pigments present within the deeper portion of shell. The relationship is likely to be indicative of pigment made available by the shell gland throughout the latter stages of egg shell formation. Cuticle pigmentation is therefore more closely related to reflectance than reflectance is to true shell pigment content.

Egg size and cuticular mass are weakly related with true shell pigment but fail to correlate with cuticular pigment. Large eggs appear to contain more shell pigment and cuticle than smaller eggs although this relationship with colour was not apparent when egg size and reflectance were compared in Section 4.1. Intensity of colour, however, fails to increase, in parallel with the raised pigment content in large eggs.

Shell colour (as indicated by reflectance) is closely associated with other egg shell factors such as its weight, its shell and cuticle pigment and also the mass of the proteinaceous cuticular layer. The colour intensity of the domestic hen's egg shell is a product of multiple factors, all of which require to be considered when colour quality is considered.

4.4 THE EFFECT OF AGE ON THE DEPOSITION OF CUTICLE AND ITS ASSOCIATED PIGMENT CONTENT

4.4.1 Introduction

Egg shell colour intensity has been demonstrated to decrease with age, using reflectometry (see Section 4.1.4). The cuticle is the major carrier of the pigment responsible for the expression of shell colour. This loss of shell colour with age, to date, has not been demonstrated chemically. Results presented in the previous section show a significant correlation between cuticular mass and its associated pigment content at one point (26 weeks) in the laying period. However, it is not known whether changes in cuticle mass with age take place, thus playing some role in influencing the associated pigment content. The cuticle plays an important role as a primary physical barrier to liquid and particle (including microorganisms) penetration of the pores (Board, 1974). Egg shell cuticle is also an important component in a series of resistances associated with gaseous diffusion across the egg shell (Deeming, 1987). Changes in the degree of cuticular cover may therefore also have important implications to both eggs for marketing and breeding purposes. Several investigations have shown that changes occur in the egg shell characteristics of the

domestic hen throughout the laying period, e.g. Hamilton, (1978); Perek and Snapir (1970), Roland (1976) and Nys (1986). A more detailed and systematic study, is however required to investigate the effect of age on the deposition of cuticle and its porphyrin content in a commercial laying strain of domestic hen.

4.4.2 Aims

To investigate the effect of age of hen on the deposition of cuticle and associated protoporphyrin-IX content using the methods developed earlier, with eggs from the test flock.

4.4.3 Experimental procedures

Twenty two hens were selected at random from the test flock. The hens and cages were individually marked and eggs were collected from each hen every ten weeks between 30 and 90 weeks of age. All eggs were weighed and a reflectance measurement recorded from the broad-poles. The cuticles were individually removed from each egg shell as before, and hydrolysed in the HCl-HC00H (5%) mixture for 22 h at 94°C. Pigment and amino acid content of each cuticle were estimated as described previously.

The mean reflectance, cuticular amino acid and pigment content were calculated for each of the 10-weekly samples. Mean values were compared with those of week 30. The change in each estimation against age was subjected to regression analysis. The significance of the regression model was calculated using the method of least squares analysis (Steel and Torrie, 1980).

Table 4.9
Mean values of reflectance, cuticle mass and protoporphyrin-IX
content of egg shells from hens between weeks 30 and 90

Measurement	age weeks						
	30	40	50	60	70	80	90
egg shell reflectance (%)	34.88 (5.03)	35.67 (5.86)	36.63 (6.93)	37.67 (6.07)	37.36 (7.72)	37.96 (6.48)	39.33 (6.72)
change in reflectance	0	0.79	1.75	2.79	2.48	3.08	4.45
proto-IX ¹ (μg)/cuticle	52.54 (23.04)	40.62 (28.16)	38.36 (29.13)	28.76 (17.58)	26.22 (12.36)	23.09 (11.45)	22.84 (13.62)
change in proto-IX(μg)/cuticle	0	11.92	14.18	23.78	26.32	29.45	29.70
cuticle mass (mg)	15.1 (2.8)	15.2 (2.9)	14.6 (3.6)	15.1 (2.1)	16.3 (5.8)	15.4 (3.0)	14.0 (2.9)
change in cuticle mass (mg)	0	0.1	-0.5	0	1.2	0.3	-1.1

means + SDEV, n=22 eggs/group
1 protoporphyrin-IX

Table 4.10

Coefficients of regression equations¹ for egg shell measurements between
30 and 90 weeks of age

Test (X)	Measurement (Y)					
	Shell colour (%)			Cuticle porphyrin (μg)		
	a	b	r ²	a	b	r ²
Age	33.07	0.0666	0.967+0.05**	62.41	-0.487	-0.951+0.07**
Change with age	-1.525	0.0628	0.947+0.09**	-2.931	0.391	0.951+0.09**
Change in shell colour with change in cuticle porphyrin		a	b		r ²	
		-0.679	0.143		0.892+0.17*	

1 Equations in the form $Y=a+bx$, a=intercept of measurement, b=slope

2 Correlation coefficient + 95% confidence limits

* $P < 0.05$

** P<0.01

4.4.4 Results

Mean values (\pm SDEV) for measurements of egg shell reflectance, cuticle mass and protoporphyrin-IX from eggs (n=22 per group) at ten weekly intervals from 30-90 weeks are presented in Table 4.9. Age had a considerable influence on most measurements. Both reflectance values and porphyrin content of each sample was variable, with large standard deviations, i.e. 34.88 ± 5.03 to 39.33 ± 6.72 and 52.54 ± 23.04 to 22.84 ± 13.62 between values from 30 and 90 week samples respectively. As egg shell reflectance increases with age, there is a corresponding loss of cuticle pigment. When the data were analysed by the method of least squares analysis, it most closely fitted linear regression models as shown in Table 4.10. Both reflectance and cuticle pigment was highly and significantly correlated with age, viz: $r=0.967 \pm 0.05$ and -0.951 ± 0.07 ($P<0.01$) respectively. Cuticle pigment is negatively correlated indicating it loses pigment with age. Reflectance is related positively to age but, negatively to shell colour, therefore colour intensity decreases with age. When the changes in reflectance and cuticle pigment with age were compared with those of week 30, there was a similar highly significant and positive linear relationship ($P<0.01$). The change in reflectance with changing cuticle pigment content showed a significant and positive relationship ($P<0.05$). Cuticle mass did not significantly change with age and no correlation with either reflectance values or cuticle pigment was observed.

4.4.5 Discussion

The results show that as the domestic hen ages, shell colour intensity decreases and there is a corresponding loss from the egg

shell of porphyrin pigment, which is expressed in the cuticle. Cuticle pigment estimations therefore are a suitable indicator of the overall reduction in shell pigment with age. The reflectance increase observed over the laying period compares favourably with that observed in Section 4.1.4 with the larger flock. Analysis of the regression equations indicate that shell colour intensity is lost at a rate of around 0.67 reflectance units every 10 weeks. This corresponded to a decrease in porphyrin content of $4.9 \mu\text{g}$ per cuticle over the same period. A 2% loss of cuticle pigment reduces shell colour by about 10% which indicates that relatively small changes in pigment deposition with age affect shell colour. Egg weight has been demonstrated to increase significantly at a higher rate than shell weight with age (Perek and Snapir, 1970). It was shown that hens produce thinner egg shells with age because their eggs become larger, but shell weight remains fairly constant, Roland (1976). However, these results indicate that as the hen ages, shell colour intensity decreases but there is actually a reduced deposition of porphyrin pigment, therefore the decrease in colour intensity does not reflect solely a thinner dispersion of pigment over the larger egg. Nevertheless, due to a larger egg size with age the thickness of the already reducing pigment deposit may contribute in some way to a lowering of shell colour intensity with age. Age, therefore appears to be the major factor causing a decrease in the hen's physiological capacity to synthesise or transport porphyrin to the egg shell.

The results presented in Section 4.3.4 have shown that egg size and cuticle mass are positively correlated. Larger eggs therefore possess more cuticle. However, studies of cuticle in this investigation

agree with the results of Ball et al. (1975) and show that cuticle mass does not significantly change throughout the laying period. The results are also in keeping with those of Roland (1976) who demonstrated that egg shell weight remains fairly constant with age. Due to increasing egg size, with age, it may be expected that cuticular depth would decrease as it is deposited on a larger egg size. This decrease in the thickness of the cuticle would contribute to the reduction in overall shell thickness. Cuticle significantly influences shell thickness (Belyavin and Boorman, 1980) and any increase in egg size would result in reduced cuticle depth; this agrees with the form of loss in shell thickness observed by Roland (1976), with age.

Cuticle mass is positively correlated with its associated porphyrin content (see previous section). However, the results show that changes in cuticle pigment with age are independent of cuticle mass. Cuticle mass, is therefore more closely associated with shell weight characteristics with age than it is to shell pigment content. The method of Ball et al. (1975) using the protein stain Edicol Pea Green to demonstrate cuticle on egg shell with age, is largely subjective and intensity of staining probably indicates more closely variations in cuticular depth (which varies around 10 μ m; Simons, 1971) rather than total cuticle mass. Observations in this study using Edicol Pea Green Stain (1% v/v) to indicate cuticle on the broad-pole of the egg shell showed staining intensity to be variable but was not significantly different when compared to other portions of the shell. The accuracy of the method was enhanced when reflectometry was used to measure the intensity of stain on the broad-pole. Results indicate that the stain in conjunction with a reflectance measurement may be a

suitable method for assessing changes in cuticle depth, such as identifying cuticle-less eggs, in a commercial environment. Analysis of cuticle and pigment in greater detail, however, requires cuticle isolation, to monitor porphyrin content, with respect to investigating egg shell quality with age. It has been suggested that the loss of shell pigment with age may be as a result of reduced egg retention within the shell gland of ageing hens during the final stages prior to oviposition. Cuticular mass, however, remains unchanged with age, so it is reasonable to assume that the pigmentation process is also fully complete before oviposition takes place.

The results show that the reduction in shell colour is due to a loss of egg shell porphyrin pigment (as protoporphyrin-IX) which is expressed in the cuticular layer of the egg shell. Measurement of cuticular pigment was found to be closely representative of the decrease in shell colour intensity. As cuticle mass remains constant with age, it may contribute to thinning of the shell, hence reducing egg shell quality in eggs from older hens. Pigment loss from the cuticle appears to be mainly independent and probably represents the hen's reduced ability to synthesise porphyrin or transport it to the shell with increasing age.

4.5 THE TIMING OF EGG SHELL PIGMENTATION AND CUTICLE FORMATION IN
THE DOMESTIC FOWL. QUANTITATIVE STUDIES ON THE PROGRESSIVE
DEPOSITION OF CUTICLE, ITS PORPHYRIN CONTENT AND LEVELS OF
SHELL GLAND TISSUE PORPHYRIN PRIOR TO OVIPOSITION

4.5.1 Introduction

The formation of the hen's egg shell within the shell gland pouch occupies the majority of time (approximately 20 h) the egg spends in the oviduct of the domestic hen (Warren and Scott, 1935). The processes involved in shell formation have been exhaustively documented (see Section 1.1. for a resume of these processes). Consequently, research towards understanding the secretions from the shell gland have largely concentrated on the formation of the true shell during calcification (e.g. Burmester, 1939; Mongin and Sauveur, 1970; Talbot and Tyler, 1974 and Nys, 1986). Nevertheless, little is understood about the events which lead to the deposition of the egg shell cuticle and the pigments, prior to oviposition. Early studies by Warren and Conrad (1942) showed that in the domestic hen, pigment was deposited during the entire period of shell formation, but that the rate accelerated in the last 3 to 5 h before oviposition. They also noted that the pigment speckles on turkey eggs were deposited near the time of lay. In the Japanese quail, Coturnix coturnix japonica, incipience of superficial pigment deposition on eggs was observed by Woodard and Mather (1964) at 3.5 h and by Poole (1965) between 2-3 h before lay. The pigmentation process in the domestic fowl has not been fully described by suitable quantitative means. For example, although cuticle deposition is thought to begin after the termination of calcification, there has, so far, been no study to substantiate this;

nor has there been an investigation into the rate at which deposition of porphyrin on the cuticle takes place during the period prior to oviposition.

4.5.2 Aims

To measure the levels of porphyrin in the major regions of the hen's oviduct.

To investigate egg shell cuticle formation, cuticle porphyrin deposition, and the levels of shell gland tissue porphyrin, prior to oviposition.

4.5.3 Experimental methods

Management of hens

Fifty ISA Brown Hybrids, aged 25 weeks at the start of the study, were individually caged, and food and water were supplied ad libitum. Hens with a laying sequence of greater than six eggs were chosen to be included in the experimental pool. After caging, the hens were left to adjust for 7 days prior to the start of the investigation. The hens were maintained throughout all periods of this study under a lighting regime of 14 h light and 10 h darkness. However, when individuals were studied at extended periods prior to oviposition, the lighting schedule was advanced to present an expected oviposition time of around 3.00 pm. This practice enabled sufficient length of time within daylight hours for experimental work.

Determination of oviposition time

Laying records were examined to find birds which had laid eggs at a consistent time of day (± 15 min of the mean) for five consecutive days. By monitoring individual hens in this way it was possible to predict the expected time of oviposition on the following day, provided it was not a pause day.

Palpation of the shell gland pouch

Throughout this study it was necessary, prior to sacrifice, to determine whether an egg was present within the shell gland pouch. This was achieved by digital palpation of the shell gland to confirm the presence of an egg.

Sacrifice

Hens selected from the pool of fifty for inclusion in the study were killed by an intravenous overdose (2 ml) of sodium pentobarbitone.

4.5.4. Experimental details

1. Protoporphyrin-IX in oviducal segments

A mature laying hen (ISA Brown Hybrid) aged 30 weeks was sacrificed. The shell gland pouch contained a soft-shelled egg. The oviduct was removed from the body cavity and the egg taken from the shell gland. Each portion of the oviduct was separated viz; infundibulum, magnum, isthmus, tubular shell gland, shell gland pouch and vagina. Approximately 5 grams were taken from each region of tissue and homogenised using a pair of fine surgical scissors, as described in Section 3.4.3. From each homogenate 2 X 1 g portions were

taken and protoporphyrin-IX extracted then estimated by the methods also described in this section.

2. Studies on the formation of egg shell cuticle, its porphyrin and shell gland tissue porphyrin.

From the pool of fifty hens, all eggs were collected daily, for at least five days (for use as controls) prior to commencement of the experiment. The eggs were weighed to the nearest 0.1 g and the broad-pole reflectance recorded. All eggs were stored in the dark until required. Individual hens from the pool demonstrating regular oviposition intervals of 24 h, over at least 5 consecutive days (as described earlier) were chosen for inclusion in the study. Suitable hens were palpated, to confirm the presence of a hard-shelled egg within the shell gland. Five hens were randomly sacrificed at each of the following intervals after the previous oviposition, viz; 15, 18, 21 and 23 h (i.e. 9, 6, 3 and 1 h prior to expected oviposition) respectively. The shell gland, containing the premature egg, was separated from the oviduct and immediately placed on ice. The egg was carefully removed from the gland by cutting along the longitudinal axis of the tissue to expose the egg within. After weighing the egg, a broad-pole reflectance measurement was taken, and the egg stored in darkness until required. The weight of the empty shell gland was recorded and the tissue stored at -70°C . Tissue protoporphyrin-IX was estimated by the method described in Section 3.4.3. The cuticles from both control (n=5 eggs from 5 hens/time interval) and premature (n=5 eggs at each time interval) eggs were removed as described in Section 3.2. Cuticle mass and its associated porphyrin content were measured in all samples by the methods described in Sections 3.5 and 3.4.1 respectively.

The mean values of measurements of reflectance, cuticle mass and cuticle porphyrin were calculated for all groups of eggs. The differences between control and premature values were calculated. Data were subjected to analysis by Student's t-test and linear regression analysis.

4.5.5 Results

1. Oviducal protoporphyrin-IX

The results presented in Table 4.11 show that all the major regions of the domestic fowl's oviduct contain detectable amounts of the pigment protoporphyrin-IX. The shell gland pouch, which auto-fluoresced with greatest intensity, contained the highest concentration, 8.43 $\mu\text{g/g}$ tissue, of protoporphyrin-IX of all regions. Both the tubular shell gland and vaginal tissue contained relatively high concentrations of the porphyrin in comparison with tissue from the infundibulum, magnum and isthmus, which contained negligible ($<0.5 \mu\text{g/g}$) pigment. The infundibulum did, however, autofluoresce faintly, and contained slightly more pigment than the magnum or isthmus.

The laying performance of individual hens, in this study, within the test flock ($n=50$), was variable. A group of hens, however, were chosen which demonstrated regular 24 h laying cycles. Other individuals layed at very irregular intervals.

The results shown in Table 4.12 demonstrate that the mean values of reflectance, cuticle mass and cuticle porphyrin of control eggs varied from group to group as was expected. This is due to the variations encountered when estimations are made from individual hens

Table 4.11
The protoporphyrin-IX content of the major regions of the hens oviduct

Section of the oviduct	protoporphyrin-IX content ¹ (μ g/g tissue wet weight)	macroscopic visual assessment of fluorescence ²
shell gland pouch ³	8.43	extremely strong
vagina	1.89	marked
tubular shell gland	2.38	slight
infundibulum	0.25	slight
magnum	0.13	none
isthmus	0.24	none

- 1 Means based on two replicates
2 The intensity of tissue fluorescence when a portion was irradiated by u.v. light
3 The shell gland pouch contained a soft-shelled egg expected to be oviposited within 15 h.

Table 4.12

Reflectance, cuticle mass and cuticle porphyrin values of normal and eggs prematurely taken from the shell gland at intervals from the previous oviposition

Hours from the previous oviposition	Control eggs ¹			Premature eggs ²			Differences			Percent deposited
	Cuticle			Cuticle			Cuticle			
	Egg shell reflectance	Mass (mg)	Porphyrin (μ g)	Egg shell reflectance	Mass (mg)	Porphyrin (μ g)	Egg shell reflectance	Mass (mg)	Porphyrin (μ g)	
15	36.4 (2.30)	18.35 (0.97)	45.13 (7.84)	75.5 (1.71)	2.51 (0.58)	0.99 (0.30)	39.10 (1.39)	15.84 (0.84)	44.14 (7.56)	13.7 2.2
18	34.5 (1.57)	14.69 (1.29)	32.11 (6.13)	70.2 (2.19)	4.29 (0.63)	2.59 (0.56)	37.78 (2.29)	10.40 (0.88)	29.51 (6.61)	29.2 8.1
21	38.7 (1.50)	14.94 (2.18)	35.12 (5.48)	65.1 (3.30)	10.98 (2.18)	6.63 (1.29)	26.32 (3.93)	3.95 (0.44)	28.49 (6.25)	76.6 18.9
23	36.3 (2.41)	17.94 (2.23)	68.37 (12.87)	39.0 (1.68)	16.40 (2.22)	50.10 (7.27)	2.68 (0.84)	1.59 (0.31)	18.27 (7.69)	91.2 73.3

¹ Mean \pm SEM based on 25 normal eggs in each group.

² Mean \pm SEM based on 5 premature eggs removed from the shell gland at intervals from the previous oviposition.

Table 4.13

Coefficients of regression equations² in which egg shell reflectance, cuticle mass and cuticle porphyrin were regressed against time from previous oviposition (i.e. 15, 18, 21 and 23 h)

				Egg shell estimation			
Egg shell reflectance (%)		Cuticle amino acid (mg/cuticle)		Cuticle protoporphyrin-IX (μg/cuticle)			
a	b	r ²	a	b	r ²	a	b
108.86	-4.28	-0.888±0.21*	43.27	-1.84	-0.996±0.08**	85.69	-2.88
							r ²
							-0.949±0.10*

1 equations in the form $Y = a + bX$, where a = intercept and b = slope, based on mean values of the difference between control and premature eggs.

2 correlation coefficient ±95% confidence limits

** P<0.001

* P<0.05

within each group. The values for premature egg shell estimations were, therefore, adjusted by comparing them to those of control eggs from the same individual. The time factors for premature eggs taken from the shell gland were estimated by the number of hours before the predicted oviposition. At each stage, i.e. 15, 18, 21 and 23 h from the previous oviposition, the values of the premature eggs were expressed as a percentage of the normal eggs.

2. Egg shell and tissue measurements prior to oviposition

Reflectance

Colour intensity of egg shells was measured by reflectometry (see Table 4.12). Between the intervals 15-18 and 18-21 hours after the previous oviposition, the eggs acquired darker shells. Reflectance readings at each of these stages, consecutively dropped by 5 units. A major decrease in premature shell reflectance of 26 units then occurred during the following 21-23 h stage. After this dramatic change the difference between control and premature shell reflectance was negligible.

Cuticle deposition

The data of amino acid deposition with time from the previous oviposition, shown in Figure 4.6 fitted a linear regression line, shown in Table 4.13, ($r=0.996$, $P<0.001$). Assuming linearity, at the 15 h stage, around 10% of the cuticle has been deposited, and by 19 h almost 50% of the cuticle had been deposited. By 1 h prior to expected oviposition, at the 23 h stage, around 90% has been laid down. The remaining 10% is deposited during the final hour the egg spends within the shell gland. When the percentage differences between the observed values are considered, there is a rapid increase in the rate of cuticle

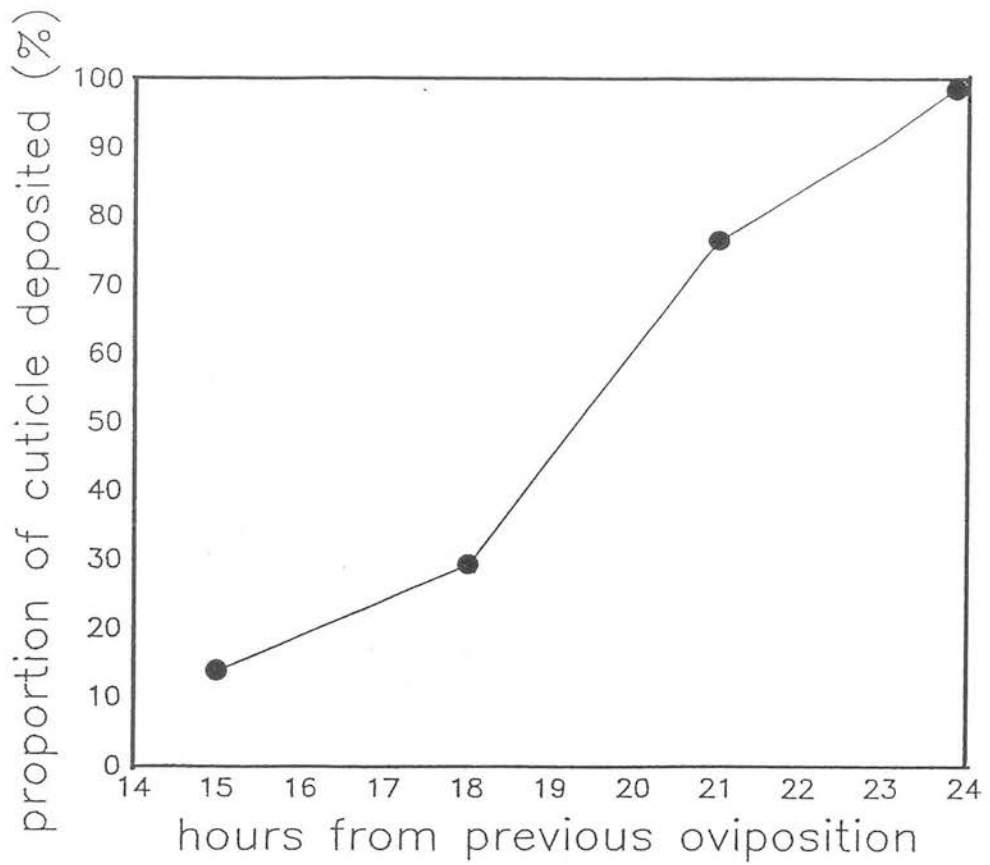


Figure 4.6 The rate of cuticle deposition (expressed as a proportion of normally oviposited control eggs) at intervals from the previous oviposition. n=5 eggs at each time interval..

Table 4.14

Shell gland protoporphyrin-IX at intervals from the
preceding oviposition

Shell gland tissue			
Hours	Weight (g)	Protoporphyrin-IX (μ g/g tissue wet weight)	total protoporphyrin-IX (μ g)
15	25.07 \pm 0.63	10.11 \pm 0.96 ^a	259.33
18	28.83 \pm 1.84	17.96 \pm 0.16 ^b	427.99
21	23.01 \pm 2.02	13.83 \pm 1.50 ^c	318.23
23	22.71 \pm 1.36	5.66 \pm 0.83 ^d	128.52
24	24.03 \pm 1.96	3.47 \pm 0.66 ^d	83.37

Means \pm SEM based on duplicate estimations from 5 hens at each time interval from the previous oviposition.

Values having different subscripts are significantly ($P < 0.05$) different.

deposition between 18-21 h stages with around 43% being added to cuticular mass. A sharp decline in the rate of deposition then follows and deposition becomes more linear during the final 3 h prior to oviposition.

Cuticle protoporphyrin-IX deposition

The deposition of cuticular pigment (see Figure 4.7) was slow but constant with time between the 15-21 h stage, with 8% of the total pigment being deposited by the 18 h stage. By 21 h a further 11% of the pigment had been deposited on the cuticle. The rate of the deposition then became more rapid between 21-23 h. At this stage a further 54% of the cuticular pigment was deposited. During the final hour before oviposition a relatively large proportion (27%) of the pigment was still to be deposited on the cuticle.

Shell gland protoporphyrin-IX

The pigment content of shell at intervals from the previous oviposition are shown in Figure 4.6. Tissue porphyrin at all stages between 15-23 h intervals (see Table 4.14) are significantly different ($P < 0.05$). The value at interval 24 h is not significantly different from the 23 h value but is significantly different from all other stages. Tissue porphyrin concentration (as shown in Figure 4.8) rises steeply in the 3 h interval between 15-18 h stages by $7.85 \mu\text{g}/\text{gram}$, to $17.96 \mu\text{g}/\text{gram}$ tissue. Thereafter, tissue porphyrin becomes greatly depleted to a level of $13.8 \mu\text{g}/\text{gram}$ at 21 h. Between the remaining 2 h, from 21-23 h stages, the porphyrin level dramatically falls by some $8.2 \mu\text{g}/\text{gram}$. During the final hour before oviposition the level continues to fall but the level was not significantly different from the tissue pigment at oviposition.

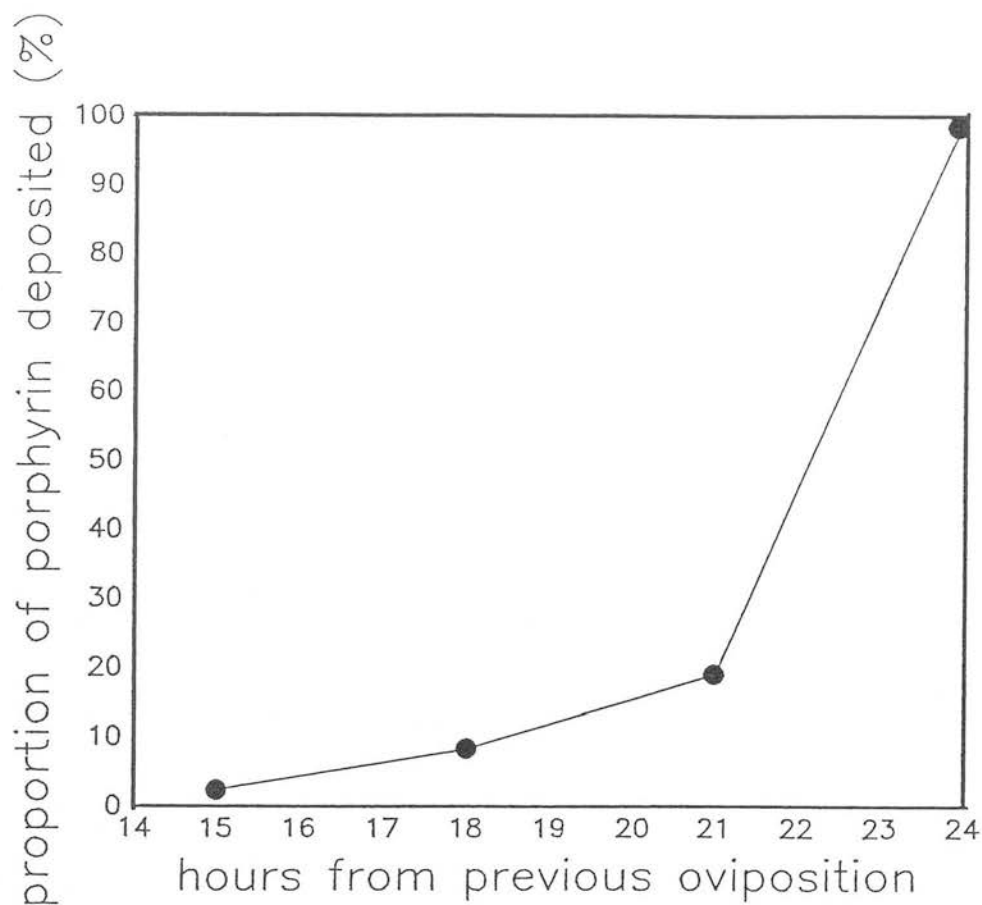


Figure 4.7 The rate of cuticle protoporphyrin-IX deposition (expressed as a proportion of normally oviposited control eggs) at intervals from the previous oviposition. n=5 eggs at each time interval.

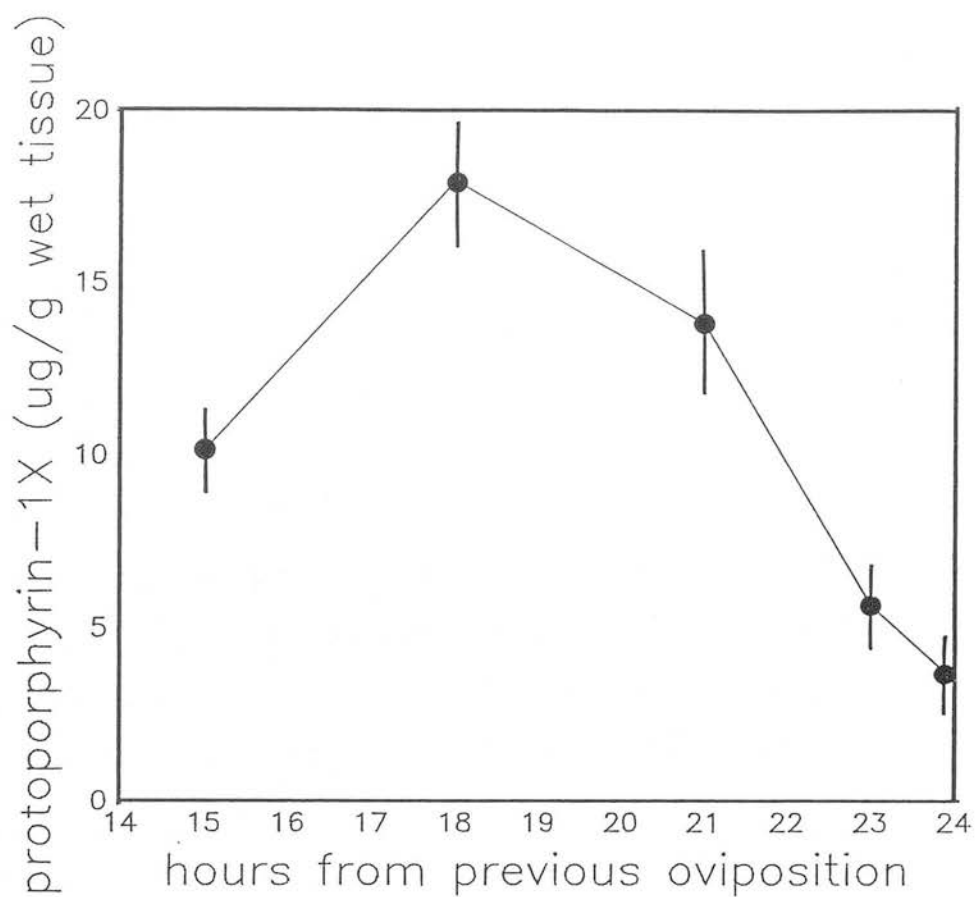


Figure 4.8 Shell gland tissue protoporphyrin-IX at intervals from the previous oviposition. n=5 hens at each time interval, mean \pm SEM.

The total porphyrin levels per shell gland, shown in Table 4.14 are based on shell gland weight, and, as would be expected, follow a similar trend to the above data, of the uptake of pigment by the cuticle.

4.5.6 Discussion

The results presented in this section have confirmed the observations of Tamura et al., (1965), that the shell gland pouch is the principal site, within the avian oviduct, which is responsible for pigmenting the egg shell. The shell gland pouch, when compared with the other major portions of the oviduct, autofluoresced intensely and contained the greatest concentration of protoporphyrin-IX. Porphyrin values of 8.5 μg per gram of shell gland tissue compared favourably with estimations by Polin (1965) of around 6.9 μg /tissue in New Hampshire brown laying hens. Tissue from the infundibulum contained significant quantities of the porphyrin when compared with tissue from the magnum or isthmus. It has been observed (J.W. Wells, unpublished) that the ovum of the fowl contains porphyrin (hence its autofluorescence). The porphyrin of the infundibulum tissue may result from transfer from the ova as they are engulfed by the tissue preceding ovulation. A sharp increase in pigment content was observed when travelling from the isthmus to the tubular shell gland. Tissue of the tubular shell gland probably contains pigment as it is passed to this region from the shell gland pouch during muscular contraction of the oviduct. The tubular shell gland is involved mainly in mamillary core formation and initial calcium deposition (Stemberger et al., 1977; Davidson, 1973). This observation is probably a result of the egg

shell depositing quantities of cuticle-pigment mass, during oviposition. Immediately following oviposition, the cuticle can easily be scraped from the shell. However, within a few minutes the mass becomes hardened and adheres firmly to the underlying true shell.

The egg shell cuticle and its associated pigment are deposited on to the egg shell of the fowl during the final 9 h prior to oviposition. The use of chemical measurements in conjunction with reflectometry to measure pigment deposition has reinforced the findings. Variation in the estimations of cuticle mass and porphyrin existed within both control and prematurely expelled groups of eggs. This variation is a characteristic of the irregular distribution of egg shell data rather than of the errors associated with the quantification of amino acid or porphyrin. The corresponding variability of reflectance data confirms the spread in colour intensity observed within the groups. Estimations of both cuticle mass and porphyrin content are highly repeatable. Deposition rates of these substances, prior to lay, within individual hens, may however be more constant.

Cuticle and pigment have been shown to be present in small quantities, some 9 h prior to oviposition. The active process of deposition therefore commences relatively early within the shell gland. The rate of cuticle formation from this 9 h point is slow until the 6 h stage. The following period to 3 h before lay, demonstrates an accelerated rate of deposition, during which the majority of egg shell cuticle is formed. During the final 3 h the rate of deposition slows down, becoming more linear with time until the point of lay. Pigment deposition however, in parallel with cuticle formation, does not occur. Between 9 h and 3 h before lay, pigment is deposited on the cuticle in

a slow but linear manner. During the final 3 h before lay a rapid increase in pigment deposition occurs and the majority of cuticular pigment (around 81%) is added to the egg shell. Shell gland tissue pigment content significantly rises, presumably due to increased porphyrin synthesis between 9-6 h prior to lay. From this point tissue porphyrin is rapidly depleted until 1 h prior to lay. The rapid loss of tissue pigment correlates with the point in time the cuticle gains the majority of its pigment. At approximately 1 h before lay around 27% of the cuticle-porphyrin remains to be added. Cuticle deposition, however, continues at a higher rate approaching oviposition than pigmentation, with only around 9% of its mass to be added during the final hour within the shell gland. The observed values of the differences between control and premature cuticle mass were more linearly related with time prior to oviposition (see Table 4.13) than pigmentation data. Assuming linearity in both cases, cuticle and its porphyrin are deposited at rates of $2.9 \text{ mg amino acid h}^{-1}$ and $2.2 \text{ } \mu\text{g protoporphyrin-IX h}^{-1}$ respectively. The rate of pigment deposition, however, between the final 3 h before lay is accelerated and the actual rate at this point is likely to be higher than the above value. At this stage it is not clear what factors control the mechanism for increasing porphyrin synthesis and whether its transport from the shell gland epithelial cells is passive or mediated by a carrier.

A recent study by Nys (1986), demonstrated that shell formation in the fowl begins 9.5 to 11 h preceding oviposition, then increases linearly for the following 13 h but the rate slows down during the final 1.5 h prior to oviposition. Nys, attributed this plateau to the formation of cuticle. However, the results presented here have shown

cuticle formation to begin at a much earlier stage. Both calcification and cuticle formation probably result from secretions from the surface epithelial cells, and Baird et al. (1980) presented evidence of the basal epithelial cells being implicated in the secretion of cuticle. Cuticle deposition is about 75% complete 3 h prior to lay but whether the rate of calcification (which is vastly reduced at this point) influences cuticle formation is not known. Results presented in Section 4.3 demonstrated that egg shell weight was significantly related to cuticular mass, which may indicate a functional relationship between the two processes. During the final 3 h prior to lay when calcification and cuticle deposition rates have decreased, the remainder of the cuticular pigment is synthesised and deposited. The results show that shell gland tissue at the early stage of around 9 h before lay contains a high concentration of protoporphyrin pigment. During the rapid rate of calcification at this point (Nys, 1986) pigment may be adsorbed and incorporated within the developing true shell. This may explain the presence of pigment within the outermost portion of the true shell in comparison to the less intensely pigmented inner shell of domestic fowl eggs.

The results of Section 4.3 show that total mean egg shell (membranes, true shell, matrix and cuticle) protoporphyrin-IX levels are around 144 μg in eggs from ISA Brown Hybrids, but are variable. By taking this figure as an overall average, at the maximum point of pigment synthesis by shell gland tissue, it can be calculated that there is enough pigment to colour approximately 3 egg shells. However, at this point it is not known what factors control colour intensity or the regulation of pigment adsorption by the egg shell, but the rate of

deposition may be important as well as the individual's capacity to synthesise porphyrin during the final three hours within the shell gland pouch. Consequently, it may be assumed that the process of shell calcification and those of cuticle and pigment formation may be functionally related.

4.6 PHARMACOLOGICAL STUDIES ON EGG SHELL PIGMENTATION AND CUTICLE FORMATION BY ADMINISTRATION OF NICARBAZIN, A PORPHYRIN-INHIBITING DRUG

4.6.1 Introduction

Several workers (Cuckler et al., 1955; Polin, 1959 and Schwartz et al., 1975) have shown that the deposition of protoporphyrin-IX on egg shell is markedly decreased when the mature hen is fed a diet containing the coccidiostatic drug Nicarbazine (Merck, Sharp and Dohme). The drug is normally incorporated into the diet of chicks, for 3-4 weeks, at a concentration of 0.0125% w/w. However, in many cases the drug has been inadvertently fed to laying hens and the loss of shell colour, sometimes from whole flocks, has been financially damaging to the egg producer. Normal shell colour returns when feeding the drug has been discontinued. Nicarbazine is a mixture of two chemicals - 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,-6-dimethylpyrimidine (HDP). Individual administration of the two chemicals has shown that DNC is more potent than HDP in reducing egg shell colour. The effects of these and other compounds on egg shell pigmentation have been described by Polin et al. (1959). It has also been demonstrated that the pigment present in "meat spots" sometimes found in eggs from brown-laying hens is mainly protoporphyrin-IX (Helbacka et al., 1958a and b). Feeding

Nicarbazin reduced the concentration (as shown by fluorescence) of porphyrin within the "meat spot".

Histochemical studies carried out by Tamura and Fujii, (1965) indicated that pigment granules present in shell gland epithelial cells were associated with cuticular secretion and that these processes occurred simultaneously prior to oviposition. Studies presented in this thesis contradict these findings and indicate that pigmentation process predominantly occurs after cuticle formation and that the processes are likely to take place independently. Kennedy and Vevers (1973), proposed that egg shell porphyrin is derived from the erythrocytes, and diffuses from the cells of the oviduct where it is deposited on the egg shell in association with the calcification process. Baird et al. (1975) showed that when hens were fed a diet containing barley dressed with methyl mercury dicyandiamide, the eggs produced had no cuticular layer. Moreover, the effect of feeding Nicarbazin in amounts large enough to reduce pigmentation or its effects upon cuticle deposition has not been investigated.

4.6.2 Aims

To investigate the effects of different amounts of orally-administered Nicarbazin on the deposition of porphyrin and cuticle in the egg shell and on the concentration of porphyrin in blood and shell gland tissue.

4.6.3 Experimental procedures

Twenty five ISA Brown Hybrid hens, aged 30 weeks, were individually caged and had food and water available ad libitum.

Nicarbazin (MSD), was administered orally (see Section 3.7 for details) in 100 mg capacity gelatine capsules at a dosage of 5, 10, 20 and 40 mg per hen (n=5 hens/group) on 4 consecutive days. Eggs were collected from all hens during a 5-day period, for use as experimental controls prior to administering the compound. All eggs laid before and after treatment were individually weighed and broad-pole reflectance recorded.

5 ml of whole blood were withdrawn from the wing vein of each hen into a heparinised syringe (for use as a pooled control) prior to treatment with the compound (see Section 3.7 for the method). Blood sampling was repeated following the administration of Nicarbazin on day 4. Haematocrit values were measured in the pooled sample of blood from untreated individuals and repeated again following Nicarbazin feeding on day 4. Twelve hours after the final administration of the compound on day 4 the hens were sacrificed and the shell gland pouches removed. Protoporphyrin-IX was estimated in shell gland tissue and whole blood samples as described in Sections 3.4.4 and 3.4.4 respectively. The cuticles were removed from all egg shells by soaking in EDTA solution and cuticular protoporphyrin-IX and amino acid content estimated by the usual procedures. True shell matrix and membrane porphyrin was estimated and the total shell plus cuticle values calculated.

The mean values of all estimations on egg shells were calculated for control and treatment groups. Comparisons were made using a one-way ANOVA and Student's t-test. Blood and shell gland tissue porphyrin concentrations were also compared by Student's t-test.

4.6.4 Results

The results presented in Table 4.15 show that egg weight remained constant while feeding Nicarbazin at all concentrations except the 20 mg/day dose. At this dose, egg weight significantly ($P < 0.01$) increased by 3.4 g, when compared to control eggs laid prior to treatment.

Reflectance measurements of eggs from treated hens were higher than control egg reflectance. This demonstrates that shell colour decreases when Nicarbazin is administered. Statistical significance was reached at doses of 10 mg ($P < 0.05$), 20 mg ($P < 0.01$) and 40 mg ($P < 0.01$) per day/hen. An increase in reflectance was observed at the lowest treatment level of 5 mg, but failed to reach significance. At each significant effective dose there was a loss of shell colour intensity of 19.4, 61.6 and 63.4% respectively. The changes were significantly correlated with dose, $r = 0.9845 \pm 0.033$ ($P < 0.05$). Plate 4 shows the change in intensity of egg shells from individuals fed Nicarbazin at the doses indicated, while Figure 4.9 shows the change in reflectance measurements, from control eggs on days 1-4 of treatment with the various doses of the drug.

Feeding Nicarbazin consequently, at all doses, significantly decreased total shell (cuticle plus true shell) porphyrin. At the lowest dose of 5 mg shell porphyrin significantly ($P < 0.05$) decreased by 22%; with the higher doses of 10, 20 and 40 mg significantly ($P < 0.01$), lowering porphyrin content by 41.1, 55.5 and 76.7% respectively.

The average shell gland tissue porphyrin of treated hens were significantly lower than the control value (10.61 $\mu\text{g/g}$) prior to Nicarbazin feeding (see Table 4.16). Individual variation was

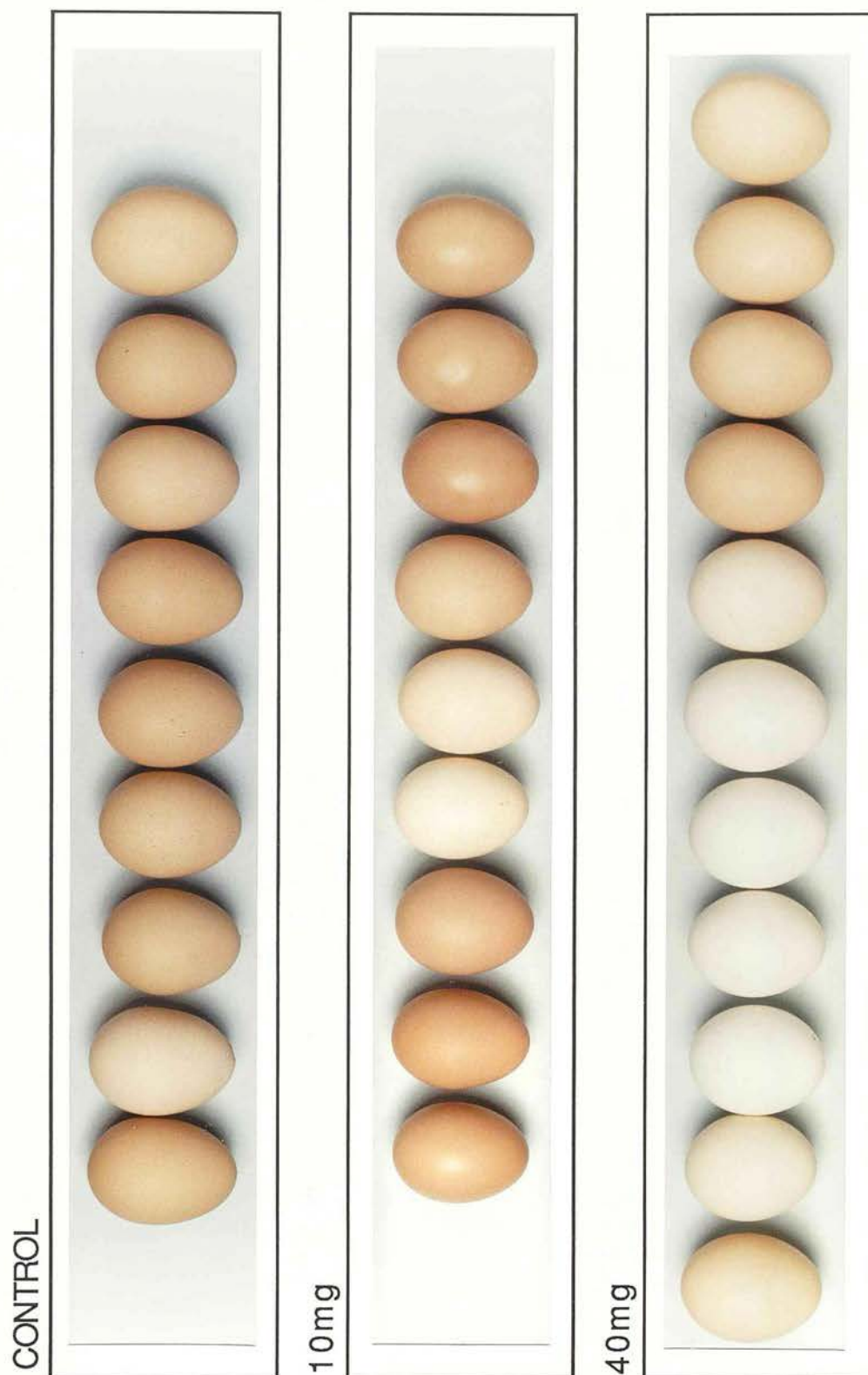


PLATE 4

The effect of feeding the drug, Nicarbazin, from day 1 to day 4 on the eggshell colour intensity of eggs from individual birds. The drug was administered following oviposition and the control hen was given an empty gelatine capsule on each day.

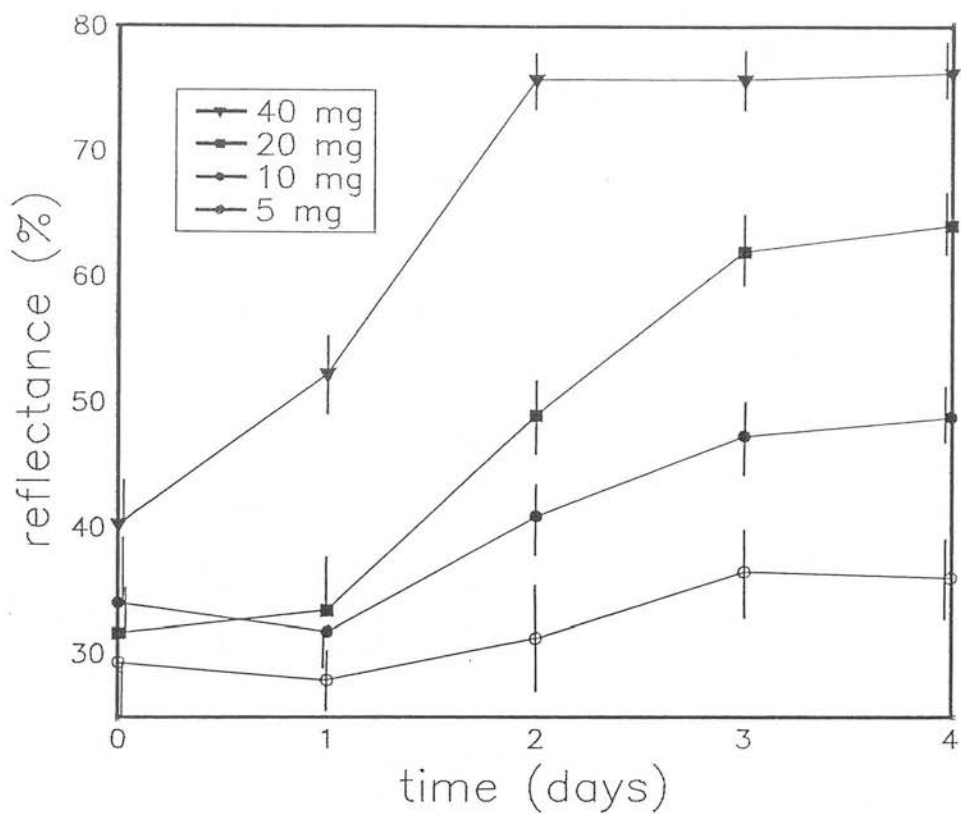


Figure 4.9 The effect of feeding varying daily doses (5 to 40 mg/hen/day) of Nicarbazin to hens from days 1 to 4 on shell colour intensity. Time zero represents control eggs prior to treatment with the drug. $n=5$ eggs per group, mean \pm SEM.

Table 4.15
The effect of feeding Nicarbazin on egg shell pigmentation and cuticle formation in domestic fowl egg shells

Estimation	Treatment group					
	Control	5 mg	Control	10 mg	Control	20 mg
Egg weight (g)	55.66 (0.67)	56.81 (1.13)	54.20 (0.72)	55.47 (0.79)	52.67 (0.67)	56.06** (0.67)
Egg shell Reflectance (%)	29.3 (1.63)	32.9 (2.07)	34.0 (1.29)	40.6* (2.32)	31.6 (1.24)	51.1** (3.14)
Cuticle mass (mg)	17.59 (0.67)	17.02 (0.92)	14.14 (0.57)	14.00 (0.68)	15.87 (0.99)	17.57 (0.89)
Total shell Protoporphyrin-IX(μ g)	161.76 (9.56)	124.63* (12.01)	140.51 (10.83)	82.67** (10.95)	117.71 (10.41)	52.39** (9.65)
					Control	40 mg
					53.12 (0.91)	54.33 (0.96)
					40.3 (2.30)	71.2** (2.37)
					16.26 (0.72)	17.15 (0.61)
					121.32 (14.06)	44.36** (7.21)

Values represent mean + SEM

Control values for each group are based on 25 eggs. Hens were administered an empty gelatine capsule on 4 consecutive days.

Treatment values are based on all eggs (n=20) following treatment. Hens were administered the dose shown each day for 4 consecutive days.

** P<0.01 significance from control values

* P<0.05

Table 4.16

The effect of feeding Nicarbazin (mg)/day on shell gland tissue
and whole blood protoporphyrin-IX concentrations

Tissue protoporphyrin-IX	Treatment group				
	Control	5 mg	10 mg	20 mg	40 mg
Shell gland ($\mu\text{g/g}$)	10.61 (1.23)	4.84** (0.53)	5.85** (0.76)	4.30** (0.54)	4.12** (0.49)
Whole blood ($\mu\text{g/ml}$)	3.16 (0.40)	5.50 (0.56)	4.77 (0.55)	5.53 (0.64)	5.78 (0.61)

Values represent means + SEM

Control values are based on 5 shell glands and blood taken from hens administered an empty gelatine capsule on 4 consecutive days.

Treatment values are based on 5 shell glands and blood taken from hens fed Nicarbazin each day for 4 consecutive days.

** $P < 0.01$ significance from control values

* $P < 0.05$.

relatively high and porphyrin levels were not related to a specific stage in the laying cycle at time of sacrifice. Increasing the dose of Nicarbazin above 5 mg failed to significantly change the concentration of tissue porphyrin. Whole blood porphyrin concentration of treated hens was slightly raised (see Table 4.16) in comparison with the pooled sample of control blood taken from individual untreated hens. The increase, however, was not significant ($P > 0.5$). Throughout the treatment period, haematocrit values remained fairly constant, except for 3 individual hens fed 40 mg/day where haematocrits following day 4 had fallen by around 15% when compared to the pooled blood average of 0.25, from normal hens.

Cuticle mass, as estimated by amino acid measurement, throughout the treatment remained fairly constant, at all dosages, and did not significantly change from control values from eggs laid prior to Nicarbazin administration.

4.6.5 Discussion

The present studies have provided quantitative evidence of the effect Nicarbazin exerts on egg shell colour intensity. The results confirm the observations of Cuckler et al. (1955); Polin (1959) and Schwartz et al. (1975), that when the drug is given to brown laying hens, egg shell colour is markedly reduced. The lowest dose which significantly reduced shell colour was 10 mg Nicarbazin per day. Colour was reduced by 19% but a larger dose of 40 mg lowered colour intensity by 63%. Polin et al. (1959) found that 10 mg Nicarbazin per day, administered intramuscularly, elicited a response but the changes observed by subjective assessment were not compared statistically. In

the present study, reflectometry detected a change when 5 mg Nicarbazin was given but the results failed to meet statistical significance. At the largest dose of 40 mg egg shells were virtually depigmented within 2-3 days. This dose most closely represents the daily intake when the drug is mixed in the diet, assuming a daily food consumption of 100-125 g per hen. The loss of shell colour at all dosages was obvious within 2-3 days, but after withdrawal of treatment shell colour returned to normal, as judged by mean values of reflectance, within 4-5 days. The increasing loss of shell colour with larger dosages corresponded to the reduction in total shell protoporphyrin-IX by up to 77%, when measured chemically. At the lowest dose of 5 mg Nicarbazin/day there was a significant reduction in egg shell porphyrin content which was not detected in shell colour measured by reflectometry. Increasing the dose led to further reductions in egg shell porphyrin. The compound is thought to reduce the production of porphyrin by enzyme inhibition at some stage in the biosynthesis (Schwartz et al., 1975). In the hen, this effect is expressed by the reduction in free protoporphyrin available to colour egg shells.

Cuticle deposition (as indicated by its mass) on egg shells regardless of Nicarbazin dosage remained unchanged. The processes of cuticle and pigment deposition probably occur independently as was indicated by normal cuticle formation, when porphyrin synthesis was pharmacologically reduced. The association of most of the organic material, which forms the cuticle and that of the porphyrin pigment, probably only takes place on the egg shell's surface when they combine to form the final egg shell deposit before oviposition.

Shell gland tissue porphyrin was reduced by up to 60% with all treatments of Nicarbazin. This is in agreement with the reduction observed by Schwartz et al. (1975) when they fed the drug to Rhode Island Red hens incorporated in an ad libitum diet. Increasing the dosage in the present study from 5-40 mg/day failed to change the degree of tissue depigmentation. Erythrocyte porphyrin at all doses, was slightly elevated by around 2 $\mu\text{g/ml}$; this confirms the results of Schwartz and co-workers, who observed an increase of 2-4-fold, when their hens were treated for a longer period of 10-13 days. The elevated blood and reduced shell gland tissue porphyrin may be taken as evidence to support the hypothesis that the shell gland is the site of egg shell porphyrin synthesis in the hen. The situation, however, is not clear. Assuming a total blood volume of around 6.6 ml/100 g body weight (De Shazer and Weiss, 1963) in the mature laying hen, sufficient free protoporphyrin-IX (400 $\mu\text{g/vol}$) is available in the blood to pigment the egg shell both before and after treatment. However, when Nicarbazin is fed, egg shell colour is reduced and the blood contains sufficient quantities of porphyrin to pigment the egg. At this point, shell gland tissue porphyrin is dramatically lowered, but the rise in erythrocyte porphyrin at this time fails to correlate with the loss of shell gland porphyrin. If the blood is the site of synthesis Nicarbazin must, therefore, reduce the ability of the blood to off-load porphyrin to shell gland tissue or for the tissue to assimilate the porphyrin from the blood. With (1973), has suggested that the shell gland tissue itself must be the site of synthesis because the egg shell itself contains significant amounts of other porphyrins such as coproporphyrin and uroporphyrin which are precursors to the formation

of protoporphyrin, therefore indicating porphyrins are not solely derived from haem degradation and therefore are probably a result of the tissue synthesising the porphyrin. Further support for the shell gland tissue comes from Solomon (1985), who suggested that although the epithelial cells of the shell gland are poorly equipped with mitochondria (the sites of cellular porphyrin synthesis), the tubular gland cells, underlying the epithelium, display a fluctuating population of mitochondria during shell formation. The tubular gland cells lack autofluorescence, which led Solomon to propose that they may be implicated in the production of the porphyrinogens, which are colourless. Auto-oxidation of these compounds when transferred to the epithelium may produce the porphyrin pigment which would then be available to colour the egg shell. The fluctuating mitochondrial populations, however, were not investigated in detail during the pigmentation process and their fluctuating numbers may not be solely attributed to porphyrin synthesis within the cell. Kennedy and Ververs (1973), proposed that pigments are derived from the red blood cells and diffuse from the blood to the oviduct to be deposited on the shell in association with calcification. This hypothesis that calcium may act as a carrier for porphyrin appears unlikely in view of the results presented in Section 4.5 which show that the rate of the pigmentation process accelerates in the last 3 h prior to lay, at which point the rate of calcification has decreased rapidly (Nys, 1986).

The activity of ALA-synthetase (see Figure 1.3) has been shown to be inhibited by Nicarbazin (Schwartz *et al.*, 1975); this led these authors to suggest that a block in the transfer mechanism of porphyrin from the blood to the shell gland tissue is unlikely, indicating that

if enzymes present within the tissue are inhibited, the shell gland itself may be responsible for porphyrin synthesis. However, the rise in erythrocyte porphyrin found in this study and the work by Schwartz et al. (1975) following treatment has still to be explained. It may also be proposed that egg shell pigments may be produced as a result of shell gland synthesis with additional porphyrin being supplied in some way by the blood.

Further studies on the effect of Nicarbazin on ALA-synthetase and other enzymes of the pathway in both blood and shell gland tissue during the pigmentation process are required to present a clearer understanding of the findings.

4.7 A STUDY OF CUTICLE MASS AND ITS ASSOCIATED PORPHYRIN CONTENT IN EGG SHELLS FROM A RANGE OF DOMESTIC SPECIES OF POULTRY. STUDIES ON THESE FACTORS IN CALCIUM "SPLASHED" EGGS EARLY IN THE LAYING CYCLE FROM A DOMESTIC HEN

4.7.1 Introduction

Kennedy and Vevers (1975), carried out an extensive survey, using spectroscopic and chromatographic techniques to identify the major pigments in wild birds' egg shells. Unfortunately, they did not isolate the cuticle, and its importance as a carrier of shell pigment was not recognised at that time. Indeed, the study described in this section is the first in which cuticle mass and its associated porphyrin content have been measured in eggs from species of poultry other than Gallus domesticus. The latter species was represented by three strains viz; ISA Brown (a commercial hybrid laying brown eggs); Ross White (a commercial hybrid laying white eggs) and Maran (a pure breed laying

dark brown eggs). Japanese quail (Coturnix coturnix japonica) which produce cryptic eggs, were included because of their economic importance in Japan (Itoh et al., 1981), France and Italy (Risse, 1980) and their increasing importance in the United Kingdom. Commercial strains of White Turkey (Meleagris gallopavo) and White Duck (Anas anas) were also included.

The second study in this section is of a rather different nature and concerns the abnormality known as "calcium splash". This is a coating of white calcareous deposit on the shell surface, sometimes seen at the beginning of lay. The abnormality is fairly common in eggs from young hens and has been recognised for many years. It is, of course, much more noticeable now that brown eggs dominate the market. There are other circumstances in which such coatings can occur, particularly in eggs from more mature hens and they will be discussed later. The results in the present study are confined to a series of affected eggs taken from a single pullet which had only been in lay for a short time.

4.7.2 Aims

To investigate cuticle and porphyrin content in eggs from a range of domestic poultry. To study these factors in relation to calcium "splashed" eggs obtained from a pullet early in the laying cycle.

4.7.3 Experimental procedures

Twelve eggs were collected, immediately after lay, from each of the following breeds of poultry, viz; ISA Brown; Ross White; Maran;

Japanese quail; White Turkey and White Duck. The eggs were weighed and a broad-pole reflectance measurement taken. The eggs were soaked in EDTA solution for 2.5 h and cuticle removal carried out by the usual method. Cuticle mass and protoporphyrin-IX content were measured as before in the egg shells, following hydrolysis in the HCl-HCOOH (5%) mixture.

Additionally a series of eggs (n=12) were collected from day 1 of lay from an ISA Brown Hybrid. These eggs were severely coated with extraneous calcium deposits. The severity of the deposit was assessed visually, the eggs weighed and the broad-pole reflectance recorded. As before, cuticle mass and protoporphyrin-IX were measured in each egg shell cuticle.

4.7.4 Results and Discussion

The results in Table 4.17 serve as a reminder of the wide range of egg size and shell colour found among different species and breeds of poultry. Reflectance values were closely related to the degree of cuticular protoporphyrin-IX content. The apparently white egg shells of the Ross White and White Duck, fluoresced weakly when irradiated under a u.v. light source. Cuticular porphyrin however, was negligible and was not detected when measured spectrophotometrically. The Maran, a pure breed, produces extremely dark brown eggs, with the lowest reflectance of all groups. Protoporphyrin-IX concentrations in the cuticles were correspondingly high, some 4-fold greater than for the ISA Brown. Selection for increased rate of lay in commercial strains, such as the ISA has resulted in a reduction in the ability to produce eggs of a dark brown colour. Cuticular masses on eggs from

Table 4.17
Egg shell cuticle mass and pigment content in eggs from a range of poultry breeds

Breed of fowl	Egg weight (g)	Broad-pole reflectance(%)	Cuticle		
			Mass (mg)	Protoporphyrin-IX (μ g)	Protoporphyrin-IX (μ g/mg cuticle)
Maran (<u>Gallus domesticus</u>)	61.06 (0.73)	11.11 (1.15)	15.36 (0.97)	219.54 (23.45)	14.29
ISA Brown Hybrid (<u>Gallus domesticus</u>)	60.18 (0.92)	33.12 (2.18)	16.66 (1.12)	58.32 (8.46)	3.50
Ross White (<u>Gallus domesticus</u>)	70.53 (1.58)	83.39 (0.75)	12.69 (0.49)	Nd	Nd
Japanese Quail (<u>Coturnix coturnix japonica</u>)	10.13 (0.21)	24.13 (2.88)	11.74 (0.39)	67.16 (8.36)	5.72
White Turkey (<u>Meleagris gallopavo</u>)	75.77 (1.97)	43.62 (3.26)	15.35 (0.90)	7.50 (1.49)	0.49
White Duck (<u>Anas anas</u>)	80.96 (1.67)	56.33 (1.34)	29.56 (0.98)	Nd	Nd

Values represent mean \pm SEM, based on 12 eggs
Nd = not detected

these two strains were similar. Eggs from the Ross White, however, contained a significantly lower mass of cuticle. This factor is probably due to strain differences rather than the colour of the egg shell. Shells from eggs laid by White Turkeys have a buff ground colour with intense dark brown speckling, concentrated mostly around the broad-pole and decreasing in intensity towards the narrow-pole. The speckles were found to be associated with the cuticle, which was demonstrated by their removal along with the cuticle when the egg was treated with EDTA solution. The pigment speckles contain protoporphyrin-IX and the degree of covering influenced total cuticle porphyrin content.

Japanase quail egg shells contain a large cuticle mass and high porphyrin content. Weight for weight, the quail eggs (around 10g) contained the highest porphyrin content per mg of cuticle in all egg shells apart from Maran. Lucotte et al. (1975), demonstrated that the quail egg shell contains high concentrations of biliverdin-IX, but the pigment present in the cuticle was predominantly protoporphyrin-IX. Also, from the present study, it is clear that protoporphyrin-IX is the predominant pigment associated with quail cuticle, with the underlying true shell containing the biliverdin-IX fraction (Lucotte et al. 1975). The association of the biliverdin (ground colour) with shell rather than cuticle suggests that two separate mechanisms may influence the deposition of each pigment. Biliverdin may be deposited during the time of calcification of the shell and the protoporphyrin slightly later in the laying cycle, preceding the termination of shell formation. This suggests biliverdin has a greater affinity for calcium in areas it occurs, and protoporphyrin is associated with the protein

Table 4.18
Egg shell cuticle mass and pigment content of egg shells coated
with post-cuticular deposits

Egg No.	Severity of calcium splash	Egg weight (g)	Egg shell reflectance (%)	Cuticle	
				Mass (mg)	Protoporphyrin-IX (μ g)
1	+++	41.62	40.9	13.1	101.55
2	++	41.68	40.6	12.6	166.60
3	+++	48.95	41.5	10.7	146.76
P 4	+++	50.04	33.4	13.2	132.15
5	+++	52.81	38.5	11.9	131.32
P 6	+++	52.26	32.8	12.0	137.57
7	++	47.62	33.7	11.0	126.67
8	++	50.13	36.3	9.8	131.26
P 9	+	50.59	33.8	10.2	131.57
10	+	52.54	30.8	13.3	98.43
11		53.11	30.2	10.6	168.32
12		52.60	28.7	11.2	103.87

P = pause day
+++ Severe
++ Moderate
+ Slight

component of the cuticle, or egg shell. Plate 5 shows the shell colour of different domestic fowl eggs which contain varying quantities of protoporphyrin-IX and biliverdin-IX.

In the second part of this study (see Table 4.18), the eggs at the start of lay from an ISA Brown Hybrid were heavily coated by calcium deposits, in the form of a fine dusting with occasional larger "splashes" of calcium. By eggs 7-8 in the sequence the severity of this covering was beginning to decrease and by egg number 11, normal eggs were being produced. At this point the egg shell colour intensity gradually became more obvious and shell reflectance was found to decrease as the amount of extraneous calcium deposit diminished. Cuticle mass and its porphyrin content remained constant as lay proceeded over egg numbers 1 to 12, indicating that although colour apparently changes, both cuticle and porphyrin deposition continues normally. The calcareous material is superficial and masks the underlying shell colour. Other abnormalities such as coatings of amorphous calcareous material ($570-580 \text{ g kg}^{-1} \text{ CaCO}_3$; J.W. Wells, unpublished) causes a change in shell colour from brown to pink (Hughes et al., 1986). This phenomenon is probably a result of light reflection from the calcareous dusting, altering the perception of normal cuticular colour on the egg shell. The major categories of these post-cuticular deposits have been called pink, chalky, dusted and white banded eggs (Hughes et al., 1986). The superficial nature of the deposits on these eggs can be demonstrated in their removal by brief immersion in EDTA solution; after washing and drying the treated egg the normal brown cuticle is revealed. Post-cuticular deposits may be due to stress on the hen. Similar deposits, as well as egg retention,



PLATE 5

eggs taken from different strains of domestic fowl which demonstrate the presence of varying quantities of the two principal egg shell pigments; protoporphyrin-IX and biliverdin-IX. The eggs at the top of the plate are caucano fowl eggs; the olive egg (left) contains a mixture of both pigments, the light blue egg (right) contains predominantly biliverdin-IX. The eggs at the bottom of the plate are from commercial strains; Ross White (left) which contains negligible pigment and ISA Brown Hybrid (right) mainly protoporphyrin-IX.

can be induced by administration of adrenaline (Hughes and Gilbert, 1984; Hughes et al., 1986).

It is not clear what factors are involved in influencing the deposition of these extraneous coatings of calcium deposits when the egg is retained. At this time cuticle and pigment deposition appear to have ceased but, in some cases when the egg is retained in the shell gland, the pigmentation process has continued (A.B. Gilbert, personal communication). Hutt and Sumner (1952), reported a case where an egg had been retained within the shell gland for several weeks. Shell deposition had stopped but the secretion of pigment had continued. This resulted in an egg which was encrusted by a 12 g coating which Hutt and Sumner demonstrated to contain mainly protoporphyrin. They noted that the crust probably also contained protein, which presumably would have been organic cuticular material. However, it appears that only the pigment content was dramatically elevated, indicating that calcification and cuticle deposition had been normal and had then ceased or slowed down. Whether the presence of the egg in a shell gland, in such a case, stimulates pigmentation or it continues in the normal cyclic manner during its retention is not clear.

CONCLUDING DISCUSSION

5.0 CONCLUDING DISCUSSION

One of the reasons for carrying out the investigations reported in this thesis was to identify problems associated with the production of eggs with a consistent dark brown shell colour. It is therefore of interest to examine the results to find out if any conclusions of practical importance to the egg producers may be drawn from them.

The initial survey, carried out on eggs from hens of a commercial laying strain, illustrated the large variability in shell colour that can occur even in such a small-sized flock. The variability was largely attributable to differences in pigmentation in eggs laid by individual hens. A disturbing feature was that, in any sample of eggs taken during the survey, there was a large proportion with pale coloured shells.

Although differences in shell colour, as measured by reflectometry, were found in sequences of eggs laid by individual hens over a short time, they were smaller than those between individuals and, indeed, were scarcely detectable by the naked eye.

The apparent increase in the flock shell colour intensity observed by week 27 in the laying period, is attributed to a reduction in the incidence of 'calcium splash' which is common among young hens early in lay. By week 29 the problem was negligible. Actual shell colour during the 'calcium splash' period was shown to be normal when these superficial deposits were removed, thus expressing the underlying true shell colour. However, as the laying cycle progressed, a decrease in shell colour was observed from the flock. The reduction was greater in eggs from some hens than from others. The mean rate of decrease in colour was 0.67 reflectance units every ten weeks from the onset of lay

until week 65. Between then and week 73 the rate slowed and thereafter only very small changes were detected. The mean decrease in shell colour over 95 weeks was 6.8 reflectance units (22% of the original value) which could be considered to be fairly low in relation to the large variability that existed at all stages. These results suggest that lack of persistency in shell colour may be less of a problem than was supposed. One national chain of retail outlets stipulates that eggs should only be marketed from birds between 35 and 65 weeks of age. It appears that age may be too crude a measurement and egg producers are penalised by not being able to take birds to the laying period which produces the greatest number of large sizes. The fact that some hens laid eggs, which maintained their colour well, may give rise to the possibility that these birds might be used for breeding purposes, although selection of individuals producing well-pigmented eggs would be the main criterion.

At this point it should be re-emphasised that unlike the yolk pigments (see Karunajewa, et al., 1984) which are derived from plant sources or by chemical synthesis, and must be incorporated in diets, the shell pigment cannot be influenced in this way and its presence on eggs is a result of its biosynthesis within the hen's body.

One factor, which adversely affects shell colour, but is controllable, is exposure of eggs to bright light. The mean loss of colour within a few weeks was as great as 47%. This finding reinforces the need to store eggs in darkened conditions although the storage period prior to marketing is considerably less than that studied within this thesis.

The comparatively recent identification of the cuticle as the main site of pigmentation was confirmed in this thesis. A similar observation was made by Baird et al. (1975), who used photography to show that the pigment in wild birds' eggs was confined to the outermost layer of the shell. Further evidence for the association between cuticle and pigment was obtained, in different ways in this thesis, viz; removal of cuticle resulted in a reduction of shell colour of about 44%; fluorescence of shell devoid of its cuticle was less intense than when the intact cuticle is present; measurement of cuticular porphyrin by chemical means and removal of eggs from the shell gland prematurely, showed the progressive deposition of pigment on the cuticle at different stages approaching oviposition. The mean mass of cuticle, as estimated by amino acid measurement, was 13.9 mg and was slightly lower than the 18 mg reported by Cooke and Balch (1969), when they isolated cuticle from a bulked sample of shells from an undefined breed of hen. The pigment associated with the cuticle and true shell in the ISA Brown Hybrid has been shown in this laboratory to be predominantly protoporphyrin-IX, although there are also traces of other porphyrins present (J.W. Wells, unpublished). Weight for weight however, the cuticle contains around 300-fold more protoporphyrin than the underlying true shell. Some 42% of the total egg shell pigment is bound to the 10 μ m coating (Simons, 1971) of mainly protein (Wedral et al., 1974) covering the egg shell of the domestic hen.

Examination of the egg shells from different species of poultry demonstrated that a wide range of colour intensity and patterning exists. Both superficial colouring and patterning were attributed to the quantity and degree of dispersal of the porphyrin pigment on the

cuticle. Cuticle mass, however, was not so variable between species as was expected. The White Duck produces large eggs, and contains around 30 mg of cuticle, whereas the eggs of the Japanese Quail, some 8-fold smaller in size, contain about 12 mg of cuticle. Other breeds had cuticular masses intermediate to these values. White eggs from hens contained less cuticle than eggs from brown-laying hens. Nevertheless, the difference is attributed to strain rather than to egg colour. Eggs from Japanese Quail weight for weight contain far more cuticle and protoporphyrin than any of the other breeds. The protoporphyrin-IX pigment is associated with the cuticle, whereas biliverdin-IX, the ground colour of the shell, is confined to the underlying true shell portion (Lucotte et al., 1975). The deposition of biliverdin would appear to occur earlier in the formation of shell than protoporphyrin, and may also have a greater affinity for calcium carbonate than does protoporphyrin, which is mostly associated with the cuticle in quail shells. Two separate mechanisms may therefore be involved in pigmenting quail eggs, but at this time the details are unknown.

Dark-shelled hens eggs in this study contained more shell by weight, than lighter eggs. This is in agreement with Grover et al. (1980) and Campo and Escudereo (1984), who demonstrated a positive relationship between shell colour and specific gravity, and shell colour and thickness, respectively. Selection for dark shells may therefore be of practical importance when wishing to improve shell strength, and may contribute to reducing the annual loss of between 5 and 7% (Peterson, 1965) associated with damage of shells. Shell weight was also related to cuticular mass and the cuticle has been shown to contribute to shell thickness by Belyavin and Boorman (1980). The

influence of the cuticle on maintaining shell strength is unclear, but shell thickness contributes to strength (Simons, 1971), so the presence of an intact cuticular layer may influence shell strength to some degree.

The loss of shell colour with age has been shown to be associated with the reduction in protoporphyrin-IX content of the cuticle. A loss of 2% in porphyrin within the cuticle has the effect of reducing shell colour (when measured by reflectometry) by around 10%. Small changes in pigment deposition as the hen ages therefore have a profound effect upon reducing shell colour intensity. These results show that, although shell deposition continues at a constant rate with age (Roland, 1976), the amount of pigment secreted on the shell is reduced. However, increasing egg size may also contribute further to this loss of colour by dispersing the pigment over a larger egg size.

Nevertheless, age appears to be the overwhelming factor causing a reduction in the hen's capacity to either synthesise or transport porphyrin to the egg shell.

The deposition of cuticle, with increasing age, similar to shell deposition, remains fairly constant, which agrees with results obtained with a subjective cuticular dye-binding method employed by Ball et al. (1975). However, the increase in egg size and the constancy in cuticle mass with age, suggest that cuticular depth may be reduced, therefore contributing to a reduction in the overall shell thickness. It is suggested that the method of Ball et al. (1975) may be a useful tool in a commercial environment, when used in conjunction with reflectometry, to assess cuticular cover on egg shells more easily

and with a greater degree of accuracy, than with the dye method alone.

When the shell gland pouch was compared to other major regions of the oviduct, it was found to contain, by far the highest concentration of the egg shell porphyrin, protoporphyrin-IX. This observation confirms the finding of Tamura et al. (1965) that the principal site within the avian oviduct, responsible for pigmenting the egg shell is the shell gland pouch itself. In this thesis, however, other regions (the tubular shell gland and vagina) were shown to contain appreciable amounts of the porphyrin. It is suggested that porphyrins may be mechanically transferred from the pouch to these regions by movement of the egg, or by muscular contraction of the oviduct.

Eggs which were prematurely removed from the shell gland pouch, some 9 h prior to oviposition, contained only small quantities of cuticular material and porphyrin pigment. The active deposition of pigment in the hen occurs some 6 h earlier in the laying cycle than reported by Woodard and Mather (1964) and Poole (1965) in quail. Warren and Conrad (1942) had earlier suggested that the pigmentation process occurs throughout the entire period of shell formation. This suggestion still holds but the results presented here show that the deposition is not constant throughout the formation of shell. At that time the existence of the cuticle had not been fully demonstrated and its importance as a carrier of pigment was not recognised. The relative increases in the rates of cuticle and porphyrin deposition were found to commence during the final 9 h the egg spends within the shell gland. During the later period between 6 and 3 h before lay the deposition of cuticular material on the egg shell is accelerated, and

around 75% is laid down during this time. Pigmentation of the cuticle, unlike cuticle formation itself, commences slowly from between the 9 and 3 h stages before lay, but the rate then rapidly increases during the final 3 hours within the shell gland. Throughout this process the depletion of shell gland tissue porphyrin inversely corresponds to the cuticle pigment content. The rate of the accumulation of tissue porphyrin increases around the 9-6 h stage, but then the rate decreases rapidly and its concentration is depleted during the next 6 h before lay in par with the increased uptake of pigment by the cuticle. The process of cuticle formation and accumulation of pigment appear to be independent, and it is suggested that as the calcification process decreases it may in some way control the point at which the majority of cuticle is formed. At this time it is not known what factors regulate the process of change from calcium to cuticle secretion. Two cell types; the basal and apical cells exist in the epithelial layer of the shell gland pouch (Richardson, 1935; Johnston et al., 1963 and Tamura and Fujii, 1966). Both types are implicated in calcium secretion (Solomon et al., 1975) and it has been suggested that cuticular deposition is a function of the basal cells of the epithelium (Baird et al., 1980).

The phenomenon of egg shells coated with extraneous deposits of calcium carbonate of various forms (Hughes et al., 1986), which cause an apparent modification in shell colour, has been shown in this thesis to have no effect on cuticle mass or associated porphyrin content. However, it is not clear why only the calcite moiety continues to be deposited during egg retention, when the pigmentation process which occurs after calcification would similarly be expected to continue.

In some cases, it has been observed that when an egg is retained within the shell gland for an extended period, pigmentation has continued, resulting in an extremely darkly pigmented shell (A.B. Gilbert, personal communication and Hutt and Summer, 1952). The egg given to this laboratory by A.B. Gilbert had a reflectance value of 7.2 units with a cuticular protoporphyrin-IX content of 0.56 mg. Egg shell weight and cuticular mass were found to be within normal values. This indicates that separate control mechanisms must exist within the shell gland pouch which regulates the deposition of pigment and each of these substances in an independent manner. Hitherto, an individual egg with an abnormally high content of shell, cuticle and pigment has not been reported.

The presence of pigment which was found in this study within the membranes and true shell portions of the egg shell may be attributed to adsorption of pigment by these portions during the early stages of shell formation, at which time the shell gland tissue contains significant quantities of the pigment. The association of the pigment with the respective portions of the egg shell is discussed later.

The drug Nicarbazin, which inhibits porphyrin synthesis (Cuckler et al., 1955; Polin, 1965 and Schwartz et al., 1975), was used as a tool to investigate the pigmentation process. Various doses of the drug reduced egg shell colour within a few days of administration. The larger the dose, the greater was the loss of egg shell colour. There was no effect on the deposition of cuticle when the drug was given, lending further support to the hypothesis that both processes of cuticle and pigment formation are independent. Cuticular mass was

shown in Section 4.3 to be related to shell weight which, along with the previous findings, by inference, supports the link of increased cuticle formation with the reduction in the rate of the calcification process. The association of organic cuticular material and its porphyrin pigment probably only takes place on the egg shell surface, when combination takes place to form the final coloured cuticular layer.

Nicarbazin treatment was found to decrease the concentration of shell gland tissue porphyrin considerably, so confirming the result of Schwartz et al. (1975). In the present study, increasing the dose of Nicarbazin failed to result in greater depletion of tissue porphyrin. Conversely, erythrocyte porphyrin at these doses was slightly raised. At this time both tissue and total blood volume contained sufficient quantities of pigment to colour an egg shell but the concentrations of each, with increasing dose, did not correlate with the changes observed. If egg shell porphyrin was blood-derived, Nicarbazin in some manner may have prevented the blood off-loading the pigment or for the shell gland tissue to assimilate porphyrin from the blood, thus explaining its elevation in blood. Nevertheless, the elevation observed here and by Schwartz and co-authors, would fail to directly correspond to the magnitude of the reduction in porphyrin deposition observed on the egg shells. The reduction in shell gland tissue porphyrin, of up to 50%, more closely corresponds to the decrease of around 77% observed in egg shell porphyrin when the drug was given. A chromatographic study by With (1973), in which porphyrins formed earlier in the biosynthetic pathway were identified in egg shell, and together with a similar study by J.W. Wells (unpublished) lend support

to the hypothesis that the gland tissue itself synthesises protoporphyrin-IX and its presence is not solely as a result of haem degradation.

Unfortunately, time did not permit the investigation of enzyme activity in relation to pigmentation, but it is interesting to speculate on some considerations of the roles they play in the process. It is recognised that the shell gland tissue contains the complement of enzymes necessary for the synthesis of porphyrin. The relative rates of the activity of ALA-synthetase (ALA-S) and ALA-dehydratase (ALA-D), (see Figure 1.3 for an outline of the biosynthetic pathway) may be used as indicators of the tissues capacity to synthesise porphyrin. The activity of ALA-S in particular, is altered in many conditions of unbalanced haem metabolism (Moore, 1980). Schwartz *et al.* (1975) found raised ALA-S and ALA-D, but reduced ferrochelatase activities in Rhode Island Red hen shell glands, (which laid brown eggs), whereas in tissue from a mutant strain (which laid white eggs), these features were reversed. Although ferrochelatase was only slightly reduced it is likely that the increased activity of ALA-S (which is subject to negative feedback inhibition by haem) makes ferrochelatase activity rate-limiting. When cellular haem concentration is low, ALA-S activity is increased, resulting in the over-production of protoporphyrin-IX and its subsequent excretion from the cell. Schwartz and Ruth (1978) termed the phenomenon in Rhode Island Red hens, laying dark brown eggs, "hereditary uterine protoporphyria" and they suggested the mitochondrial enzymes ALA-S and ferrochelatase may be located on chromosome 1 of the hen. The activity of ALA-D has also been shown to increase some 2.5-fold in quail shell gland tissue in comparison with liver,

prior to pigmentation of the egg shell commencing (Yamada, 1975). When Nicarbazin was given to hens (Schwartz et al., 1975) the activity of ferrochelatase and ALA-D were unaffected and only ALA-S activity was reduced, which indicates that the drug may exert its effect upon porphyrin synthesis (by reducing the formation of amino-levulenic acid) rather than blocking its transport to or assimilation by the shell gland tissue. ALA-S has the lowest activity in vivo, of all enzymes in the pathway, so it is reasonable to assume that the rate of the progress of the pathway depends upon its activity. The drug would therefore prevent the accumulation of free protoporphyrin-IX in the cell, thus reducing the amount of the porphyrin available for excretion from the cell, to pigment the shell. Raised levels of tissue porphyrin which were detected at around 9 h prior to oviposition may therefore be a result of increased ALA-S activity at this time. In humans, raised ALA-S is a common feature of clinical protoporphyria. Indeed it has been shown that 5 β -steroids induce the activity of ALA-S and consequently may play some role in the control of the biosynthesis of porphyrin. Similarly, Yamada (1972), showed that in immature quail shell gland tissue, the activity of ALA-D was induced by oestradiol 17- β . A hormonal control mechanism may exist in the hen, regulating an increase in the activity of ALA-S at which time a rise in tissue protoporphyrin was observed. Shell colour intensity may therefore be dependent upon ALA-S activity in the hens' tissue. The enzyme may regulate the production of free cellular protoporphyrin which may ultimately determine the degree of brownness of the egg and would explain the variation observed between individual hens. The loss of shell colour with age may, in part, correspond to a reduction in the

activity of enzymes in the pathway, thus reducing the porphyrin available to be deposited on the egg shell.

There has been little evidence presented of the mechanism by which porphyrins may be transported across the shell glands' epithelial cells before being deposited on the egg shell. Cohn (1955), suggested that the porphyrin found in the Harderian gland of the albino mouse may be transported as a porphyrin-lipid complex. However, Solomon (1983) has rejected the proposal that a similar mechanism may exist in the shell gland. Furthermore, Solomon found that there was no evidence of porphyrins complexing with ionic carriers such as calcium or magnesium. It therefore remains to be determined whether the movement of porphyrin is passive or mediated by some other carrier system.

It is interesting to speculate on the form in which pigments, after leaving the shell gland, exist within the cuticular layer. On the one hand they may simply combine or mix with the cuticle protein, thus staining the layer. On the other hand the porphyrin molecule may in some manner, form a chemical bond with the cuticular protein. An acceleration in the rate of porphyrin deposition on the shell occurs when the bulk of the cuticle has been formed. Porphyrin levels within true shell are probably low because during the time of shell formation, shell gland tissue concentration is also reduced, due to much of its content of pigment having been deposited on the previous egg. Furthermore, the formation of the cuticle on the egg may prevent additional pigment from entering the true shell by forming a barrier to further uptake; thus the cuticle absorbs a disproportionately large amount of the pigment from the shell gland. A further explanation for the localisation of porphyrin within the cuticle is proposed.

Porphyrins appear to possess strong affinities for protein in sites in which they occur in both animal and plant tissue (J.W. Wells, personal communication). The fibrils of organic matrix running through the true shell which decrease in concentration towards the exterior of the shell (Cooke and Balch, 1969b) have a protein content of at least 70% (Baker and Balch, 1962). The cuticle however, has been shown to be composed predominantly of protein (between 85-87%), by Wedral et al. (1974), and has a considerable mass of around 14 mg. Egg shell protoporphyrin may therefore be preferentially bound to the protein component, of which the cuticle has a much greater content weight for weight than the true shell. Indeed, a recent study by Mebane and Rybolt (1987) demonstrated that the uptake of food dyes, such as erythrosine, by the egg shell is dependent upon the presence of the cuticle. Cuticle-less eggs stained with less intensity than normal eggs with intact cuticles. The authors suggest an ionic bonding between the dye and the basic side groups of the polypeptide chain, and that the stability of the linkage is pH dependent. The protoporphyrin-IX molecule contains no obvious groups in which an interaction with amino acid, within cuticle may take place. Amino acid composition of cuticle protein is varied (Wedral et al., 1974) but the most likely bond may be between methyl groups on the porphyrin molecule and side groups of basic amino acids.

Further studies

Reflectometry in association with the chemical techniques which were developed, have proved useful methods for investigating egg shell pigmentation, and to monitor the decrease in shell colour as the flock aged. Furthermore the techniques enabled the cuticle to be identified

as the main carrier of pigment responsible for colour and the relative rates of cuticle and pigment deposition to be documented before lay. However, as a result of these forms of study, areas which require future investigation are proposed, which may broaden our understanding of the pigmentation process.

One possible way to investigate the formation of egg shell cuticle, prior to its deposition on shell, would be by raising an antibody to cuticle protein in an animal such as the rabbit. An initial attempt at doing this was partially successful, and an immunological response resulted (J.W. Wells and R. Burns, unpublished). By using histochemical techniques, the site of cuticle formation could be identified and its route of transfer across the cells of the shell gland would be traced.

Further studies on the pigmentation process itself are required to discover the reasons for the large variability in shell colour observed between individual hens and the lack of persistency observed in hens as the laying cycle progressed. Comparative biochemical studies on enzyme activities (such as ALA-S and ALA-D) in shell gland tissue from hens laying dark and those laying pale-shelled eggs may explain why these differences exist. Also, during the course of the research project it was discovered that viable epithelial cells of the shell gland mucosa could be isolated in large numbers by simply scraping its surface with a surgical blade. Time, however, did not permit the development of a system to culture the cells. Incubation of suspensions of epithelial cells and erythrocytes with protoporphyrin-IX precursors will indicate more clearly the synthetic capabilities of the epithelial cells compared to the erythrocytes and hence the epithelial cells role in egg shell porphyrin synthesis.

Preliminary studies also showed that ornithine decarboxylase (ODC) activity, which is stimulated when target organs are exposed to specific hormones (Cohen et al., 1970) increased several fold in shell gland tissue homogenates incubated with oestradiol-17 β . Similarly, using ODC as a marker of hormonal induction and ALA-S and ALA-D activities as indicators of porphyrin synthesis in shell gland tissue, may demonstrate whether oestrogens play a role in controlling the rate of synthesis and the deposition of porphyrin onto the shell, in a similar manner to the control they exhibit on secretions from other areas within the avian oviduct.

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APPENDIX

APPENDIX 1

EGG SHELL REFLECTOMETRY

1.1 A REVIEW OF THE DEVELOPMENT OF EGG SHELL REFLECTOMETRY

The demand for consistent shell colour from commercial laying hen's eggs has created a need for a standardised technique to measure relative shell colour. The method should, ideally, be accessible to breeders, egg producers and research workers.

Until the early 1960s, egg shell colour was measured by subjective comparisons with prepared standards (Hall, 1944; Godfrey, 1947; Farnsworth and Nordskog, 1955; Redman and Shoffner, 1961). The standards used to measure colour intensity at that time were composed of sets of 9 to 11 normal eggs with shells of varying shades from white to very dark brown (Wells, 1968). In 1962, Hunton developed an objective method to determine colour intensity by modifying an instrument routinely used in the protective coating industry to measure paint opacity (Dunkley and Collier, 1965). The instrument consisted of a photo-electric cell which measured light reflected from a surface. Hunton's method relied on the principle that brown shells reflect less light than white shells. The equipment consisted of a reflectometer head with a constant light source and a photosensitive surface which accepted reflected light. Calibration involved placing the reflectometer head on a white tile and adjusting the scale to read 100 per cent reflectance. Zero per cent reflectance was attained using the same procedure with a black tile. A direct reading of percentage reflectance could then be taken from the broad pole, the darkest area, of the hen's egg shell.

Gowe et al. (1965) produced spectral reflectance curves using a spectrophotometer with shells ranging from bright white to dark brown. These curves demonstrated that the highest resolving power occurs in the blue region of the spectrum between 400 and 500 nm. On the basis of these considerations, they inserted a blue filter in the light path of their instrument for more sensitive measurements. The procedure was highly repeatable, even with white-shelled eggs.

Earlier, Brant et al. (1953) developed a machine to grade eggs automatically for shell colour. They adapted an egg-weight grading machine to direct a beam of light on to the shell. Two photo-electric cells, one sensitive to blue and the other to the red portion of the spectrum, measured reflected light. Differences in shell colour caused a change in the ratio of light received by the photocells. The resulting signal was amplified to operate mechanical devices which separated the eggs into six colour classes. The machine was never developed commercially but it demonstrated that if the need arose eggs could be graded automatically for colour.

1.2 MEASUREMENT OF EGG SHELL COLOUR INTENSITY

Throughout this investigation, egg shell colour intensity was assessed objectively using a battery powered Sharrard Egg Reflectometer, supplied by Sharrard Developments Ltd, York.

The reflectometer head directs light through a green filter at a wavelength of 575 nm. Green light at this wavelength produces a range of readings which match visual selection of brown egg shell colour. The instrument compensates for varying external light intensities. Its portability allowed it to be used under the dull lighting within a poultry house.

Calibration

Following a warm-up period of a few minutes, the reflectometer is tuned to reference standards before use. The instrument has been precalibrated to settings equivalent to 00.0, dense black and 100.0, pure white, which represents 0 and 100 per cent reflectance respectively. The reflectometer head had to be placed on a white reference tile (supplied by the National Physical Laboratory) and calibrated to the reference figure of 78.9. The head is then placed on a black tile and adjusted to a reading of 00.0. This procedure is repeated a few times until the instrument has stabilised. A direct reading can then be taken, normally from the broad-pole of an egg. Readings may then be taken from a series of eggs in a sequence with the instrument being re-calibrated every 20 eggs.

1.3 REFLECTOMETER STABILITY TEST

Throughout this investigation, intensive use was to be made of the egg shell reflectometer. An instrument was constructed in the laboratory using an EEL mark III reflectometer head, a power charger and a Hewlett Packard Multimeter. The instrument proved to be unreliable and repeatability was low giving variable results of around 15%.

Prior to commencing work, using the Sharrard reflectometer, on a routine basis, it was considered necessary to assess the accuracy of the instrument with time. Twenty six ISA Brown Hybrid eggs were collected from each of two hens. The reflectance of each egg was measured, as described previously, at the broad-poles on the day of collection. Following the initial measurements, two further measure-

ments were repeated on the broad-poles (which had been previously pencil marked to allow a reading to be taken from the same point) at later dates of 14 and 28 day intervals. Between measurements the test eggs were stored in the dark. Some examples of the results obtained are shown below in Table 1.1

Results

The coefficient of variation with repeated measurements over the 28 day period of eggs from each hen was small, i.e. less than 0.9. This variation was considered acceptable and it demonstrated that the reflectometer was a reliable instrument to monitor shell colour intensity in this thesis, throughout the length of the domestic hen's laying cycle. An example of some of these measurements are shown below.

Table 1.1

Examples of reflectance recordings taken from
individual eggs over a 28 day period

		Time (days)			C.V
Egg No. in sequence		1	14	28	
Bird 1	1	31.1	31.3	31.1	0.37
	5	35.7	35.7	35.7	0.00
	10	30.8	30.7	30.6	0.30
	15	30.7	30.3	30.5	0.66
	20	34.9	34.6	34.8	0.44
Bird 2	1	41.1	40.9	40.7	0.49
	5	42.8	43.0	42.9	0.23
	10	40.1	40.7	40.1	0.86
	15	42.6	42.8	42.5	0.36
	20	42.2	42.2	42.1	0.14

2.

Published Work

Lang, M.R. and Wells, J.W (1987). A review of egg shell pigmentation. Worlds Poultry Science Association Journal, Vol. 43, No. 3.

A Review of eggshell pigmentation

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A Review of eggshell pigmentation

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Introduction

Egg shell colour is an important aspect of egg quality in many countries. Brown eggs presently dominate markets in the U.K., France, Ireland and Portugal, whereas white eggs are preferred in Austria, W. Germany, Switzerland and Spain (Anon, 1987), and in the United States and Australia. Shell colour has therefore been emphasised in the sales, promotion and marketing strategy of egg retailers. However, concern exists in the poultry industry regarding the variability of shell colour from brown laying flocks and the decline in intensity of colour as flocks age.

In view of the economic importance of eggshell colour as well as its biological interest, the nature of the shell pigments in various avian species is described. The biochemical and physiological processes involved in pigment formation and its deposition in and on the shell are discussed with particular emphasis on the findings obtained in studies using the domestic fowl, *Gallus domesticus*.

Pigments in Avian Eggshells

In an extensive study undertaken by Sorby (1875), the eggshell pigments from a number of avian species were subjected to spectroscopic analysis. The major pigment obtained from brown-shelled eggs was named "oorhodeine" by Sorby. Later Fischer and Kogl (1923) and Bierry and Gouzon (1939) recognized it as a porphyrin compound. "Ooporphyrin", as it was then referred to, was crystallised and later its identity was established as the haemoglobin porphyrin, protoporphyrin-IX (Fig. 1). Of more than 20 species studied, shells of all but one showed at least traces of this porphyrin. Earlier, Wicke (1858) studied the bluish-green pigment found in the shells of certain species, such as the carrion crow (*Corvus corone*) and the song thrush, (*Turdus musicus*). Krukenberg (1883) believed it to be identical to the bile pigment biliverdin (Fig. 1). Lemberg (1934) and Tixier (1945) finally confirmed its identity by isolating the compound in a crystalline form. Later, Kennedy and Vevers (1973) showed that the major pigments in the blue-green shell of the Araucano fowl (a Chilean mutant of the domestic fowl) were biliverdin, its zinc chelate and protoporphyrin-IX.

With (1973) demonstrated significant amounts of uroporphyrin and coproporphyrin in addition to protoporphyrin in the brown shells from an undefined breed of domestic fowl (see Baird *et al.*, 1975). However, work in this laboratory (J. W. Wells, unpublished) indicates that, in the brown-shelled eggs from a modern hybrid, the ISA Brown, the pigment is predominantly protoporphyrin-IX with negligible amounts of other porphyrins.

Kennedy and Vevers (1975) carried out a more extensive study to survey the shell pigments of 106 avian species. Their findings indicate that the pigments present in eggshells are protoporphyrin-IX, biliverdin-IX and its zinc chelate, and occasional traces of coproporphyrin-III. Almost 50% of the species examined by Kennedy and Vevers (1975) contained only protoporphyrin in their shells. No pigments were found in the white eggshells of the fulmar, imperial pigeon, dipper, roseate cockatoo and ring-necked parakeet. Other apparently white eggshells contained only protoporphyrin, e.g. the white stork, Barbary dove, Scops owl and roller. Others

contained both protoporphyrin and biliverdin, e.g. shells from the black-footed penguin, Humbolt's penguin, mandarin duck and wood pigeon. The species studied belong to widely separated families and there appears to be no obvious correlation between generic classification of a species and its eggshell pigment (Kennedy, 1975). Functionally, the most likely role for these pigments in wild bird shells is camouflage protection (Tinbergen *et al.* 1962; Montevicchi, 1976). However, there is also a widespread belief that brown-shelled eggs are stronger than white eggs (Solomon, 1985; Campo and Escudero, 1984), although conflicting evidence has also been reported (eg Potts and Washburn, 1974; Carter, 1975).

Biosynthesis of Eggshell Pigments

Porphyrin derivatives play an important role in the biochemistry of all living systems, indeed, they have been called the pigments of life (Battersby, 1985). The porphyrin structure is found in pigments such as the chlorophylls and haem. Porphyrins and their derivatives are also present in a wide variety of other biocatalysts, e.g., cytochromes, vitamin B₁₂ and prosthetic groups of enzymes whose biosynthesis was likely to be contemporary with the appearance of life on earth (Simionescu *et al.*, 1978).

Porphyrins comprise cyclic tetrapyrrole structures and are all related to the parent compound porphine. In nature, protoporphyrin-IX (Fig. 1) is the most common form of the fifteen possible isomers among four different possible combinations of the side chains on the porphyrin nucleus. In porphine, hydrogen atoms replace the methyl, vinyl and propionic acid side chains. Experimental

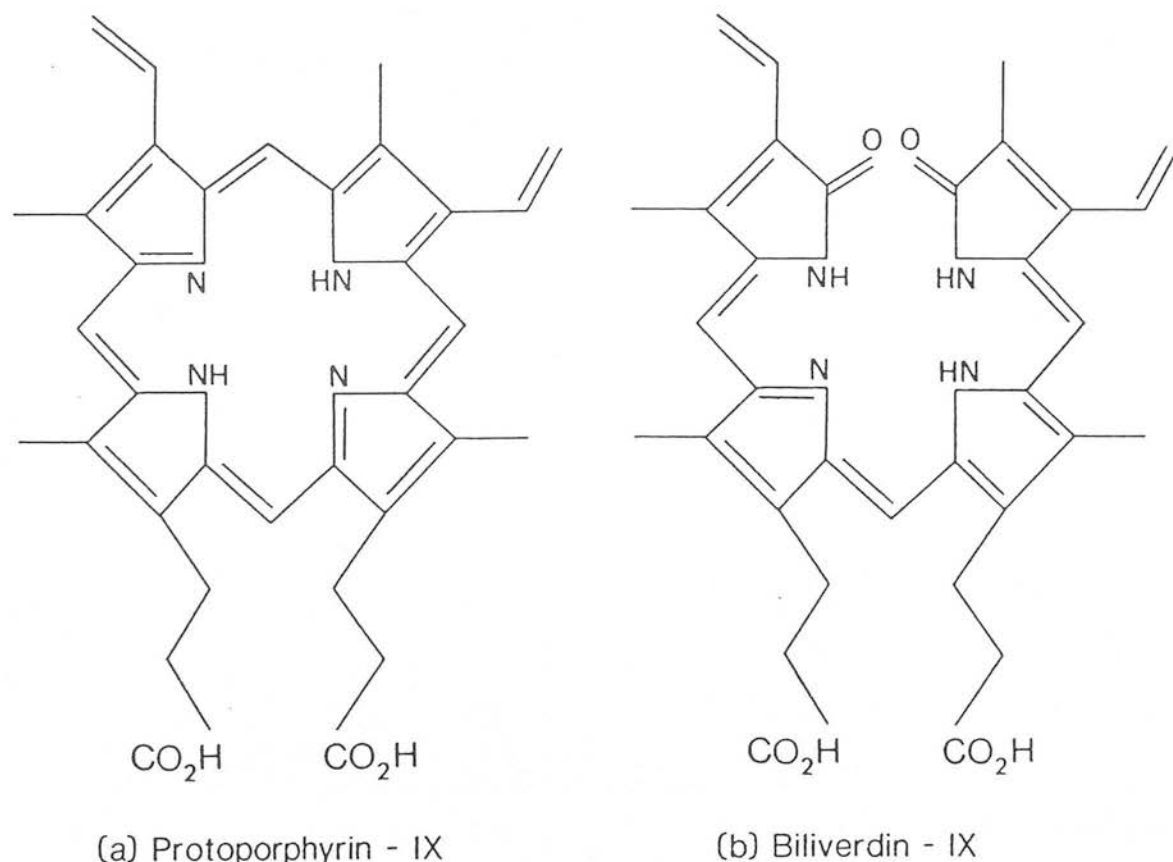


Fig. 1: Structure of protoporphyrin-IX and biliverdin-IX.

incorporation of isotopically-labelled intermediates showed that all carbon and nitrogen atoms of the porphyrin ring are derived from glycine and succinic acid.

In contrast the bile pigments are open chain tetrapyrroles. They are derived in nature by oxidative degradation and ring opening of the prosthetic groups of haemoproteins (Hudson and Smith, 1975). Rupture of the blood pigment, haemoglobin, with the loss of the meso-carbon of the methine bridge (as carbon monoxide), produces biliverdin-IX (Fig. 1). In man and mammals, biliverdin is reduced, giving bilirubin; the reduction is catalysed by biliverdin reductase.

Thus the two principal pigments of eggshells have different origins, despite chemical similarities. The porphyrins are likely to be synthesised *de novo* in the cells in which they occur, while biliverdin is probably derived from erythrocytes.

Distribution of Pigment in Eggshells

Early studies by Warren and Conrad (1942) showed that, in the domestic fowl, pigment was deposited during the entire period of shell formation, but the rate accelerated in the last 3 to 5 h before oviposition. They also found that the pigment speckles on turkey eggs were deposited near the time of lay. In the quail, *Coturnix coturnix japonica*, superficial pigment started to be deposited on eggs 3.5 h before lay (Woodard and Mather, 1964).

A seminal study by Tamura and Fujii (1967) demonstrated that in eggs from the Japanese quail and domestic fowl that porphyrin was distributed in the shell membrane, shell and cuticle. A diagrammatic representation of a radial section through an eggshell is presented in Fig 2. Schwartz *et al.* (1975) in a comparison of the

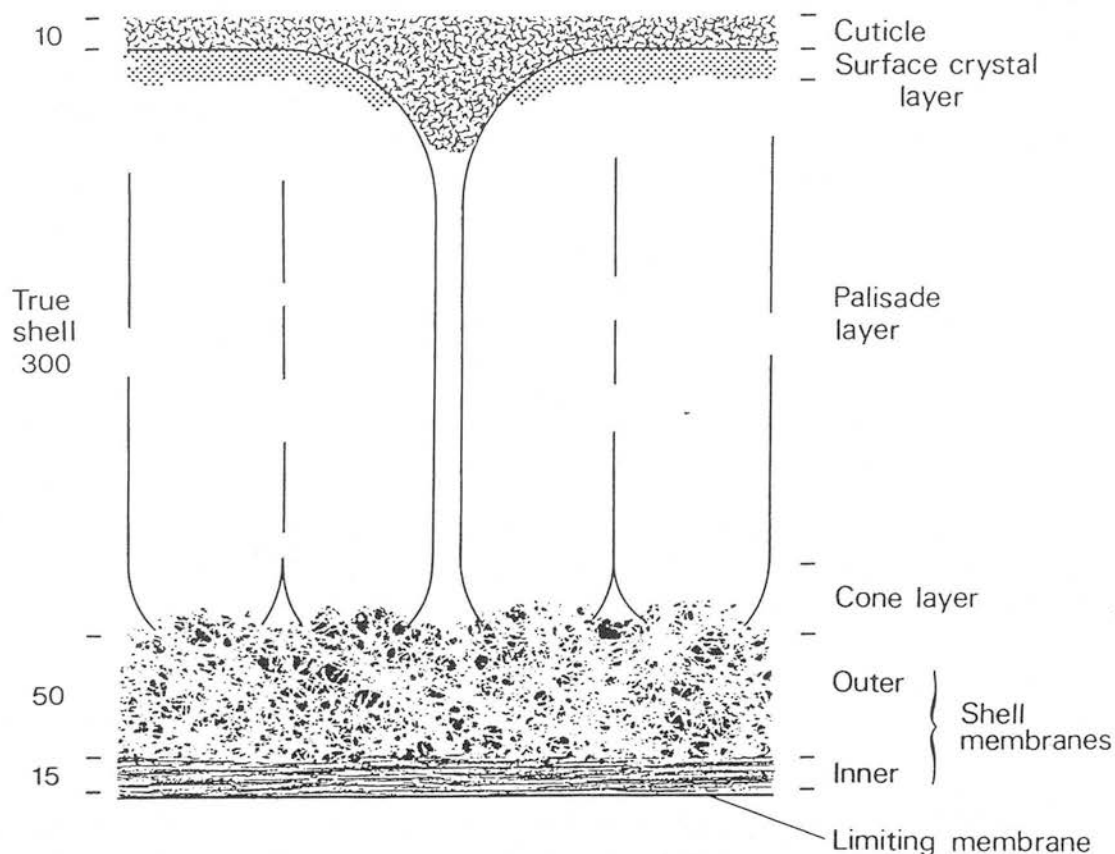


Fig. 2: Radial section through a domestic fowl eggshell showing the main structural features. Numbers on the left of the diagram are thicknesses of each layer in μm . Reproduced with permission from Tullett (1984) and Pergamon Journals Ltd.

porphyrin content of eggshells from various breeds of domestic fowl confirmed that a significant proportion of the pigment in brown eggs was in the cuticle.

These findings and our own observations (M. R. Lang and J. W. Wells, unpublished) demonstrate that the colour of eggshells from fowls laying brown eggs is due to the pigment associated with the cuticle, which is a thin (about 10^{-6} m) layer of protein combined with about 10 g kg^{-1} carbohydrate (Baker and Balch, 1962). In typical brown eggs from one commercial strain, reflectometer readings averaged 38.2% (± 1.0 , SEM $n = 47$ samples). When their cuticles were removed by soaking in EDTA for 1 to 2 h, the mean reading of the shells was 61.9% (± 1.4). Typical white eggs from a commercial strain gave a mean reading of 82.9% (± 0.4 , $n = 20$); white standard = 100% (M. R. Lang and J. W. Wells, unpublished).

Thus the cuticle acts as the carrier of pigment and the colour of eggs from domestic fowl is due to this coloured coating. The role of the cuticle in this context does not appear to be recognised by the poultry industry, although the scientific evidence has been available for over 20 years.

Origin of Shell Pigments

(a) Blood

Kennedy and Vevers (1973) considered that the porphyrins of eggshells were derived from erythrocytes which are known to synthesis porphyrins (Dresel and Falk, 1954). The arguments were based partly on histological studies by Giersberg (1921) who held that eggshell pigments were derived from disintegration of erythrocytes in the mucous layer of the shell gland.

In their 1973 paper, Kennedy and Vevers discussed theories of the pigmentation process, especially in relation to calcification, but no experimental evidence was given to support these postulates.

(b) Shell gland

Examination of the inner surface of the shell gland under ultra-violet light reveals a bright red fluorescence, confined to its epithelial cells (Tamura *et al.*, 1965). A schematic section of the shell gland mucous epithelium at various stages during egg formation is shown in Fig. 3. Tamura and Fujii (1965, 1966, 1967a, b) found small pigment granules containing porphyrin in the apical cells together with larger periodic acid-Schiff-positive granules. The abundance of both types of granules increased progressively until cuticle deposition; thereafter their numbers declined rapidly. These authors concluded that the large granules contribute to the organic matrix, while the cuticular porphyrin was derived from the small pigment granules. Analysis of tissue showed that porphyrin concentration increased during shell formation (Baird *et al.*, 1975).

Schwartz *et al.* (1980) compared the shell gland activities of several enzymes involved in porphyrin biosynthesis in normal Rhode Island Red hens with those of a mutant strain which laid chalky white eggs with negligible amounts of porphyrin. The characteristic features of the normal Rhode Island Red tissue, i.e. elevated δ -aminolaevulinic acid synthetase and low ferrochelatase, were reversed in that of the mutant. The enzymes in the shell gland of the White Leghorn hens had activities intermediate between these extremes.

This work, together with that of Polin (1957), Poole (1966), Yamada (1972) and Stevens *et al.* (1974) lends strong support for the hypothesis that the avian shell gland is the site of biosynthesis of egg shell porphyrins. However, conclusive proof must await further studies on the synthetic capabilities of the shell gland epithelial cells.

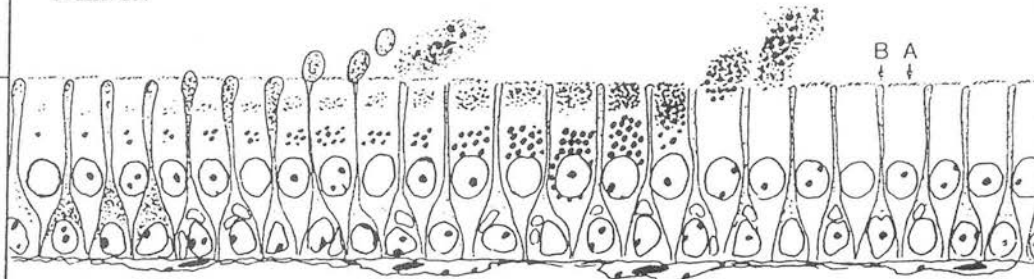
Stages	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
Position of Egg	Magnum	Isthmus	Uterus	Uterus	Uterus	After Laying
Egg covered with	Albumen	Shell-membrane		Shell	Cuticle	
Lumen	A: Apical Cell B: Basal Cell					
Epithelium						

Fig. 3: Changes in the secretion of the shell gland mucous epithelium of the Japanese quail. Reproduced with permission from Tamara and Fujii (1966).

Changes in Shell Pigmentation

Little systematic work has been carried out on the changes in the intensity of shell colour as hens age. In a flock of 96 commercial layers, the decrease in shell pigment was erratic for individual hens, but the overall changes became obvious after 39 to 40 weeks of age. At this stage, the factors causing this change are not understood (M. R. Lang and J. W. Wells, unpublished).

Disease, for example that caused by infectious bronchitis virus, is known to cause a marked increase in the incidence of pale-shelled eggs (Cook, 1986).

Similar sharp declines in shell pigment may occur through ingestion of a drug, such as the coccidiostat Nicarbazine (Schwartz *et al.*, 1975). Even doses of 5 mg per d administered to laying hens caused a change in the reflectance of brown eggs from 29.2% to 31.2% within a day, rising to 37.7% within 4 d. Higher doses lead to virtual depigmentation of the eggshell cuticle (M. R. Lang and J. W. Wells, unpublished).

With the predominance of brown eggs in the UK market, surface defects due to abnormal post-cuticular deposits which result in an apparent loss of colour have become more obvious (Van Ness, 1947). One type of deposit is the aptly named "calcium splash" seen on eggs from pullets coming into lay. The incidence of this defect decreases rapidly as egg production increases.

Another abnormality often seen on eggs from older hens is the variable superficial coating of amorphous calcareous material ($570\text{--}580\text{ g kg}^{-1}\text{ CaCO}_3$; J. W. Wells, unpublished). With some eggs, the thin coating causes an apparent change in colour to pink. The major categories have been called pink, chalky, dusted and white banded eggs (Hughes *et al.*, 1986). The superficial nature of the deposits on these eggs (or those with "calcium splashes") can be demonstrated in their removal by brief immersion in EDTA solution; after washing and drying the treated egg, the normal brown cuticle is revealed. These phenomena are associated with retention of eggs in the shell gland and may be due to the effect of stress on the animal. Similar deposits, as well as egg retention, can be induced by administration of adrenaline (Hughes and Gilbert, 1984; Hughes *et al.*, 1986).

These coatings appear different from the well-known eggshell "pimpling" which increases with age. This defect is a deposit or protuberance of calcareous material

(775 to 800 g kg⁻¹ CaCO₃) which can be reproduced experimentally by introducing CaCO₃ into the shell gland of layers (Roland *et al.*, 1975).

Conclusions

The colour of brown eggs from the domestic fowl is due predominantly to the pigment, protoporphyrin, associated with a thin layer of protein (the cuticle) coating the shell. Unlike yolk pigments (see Karunajeewa *et al.*, 1984) which are derived from plant sources or by chemical synthesis and must be incorporated in diets, the shell pigment is synthesised and secreted by the shell gland throughout shell and cuticle formation. The final coating of pigment is transferred to the cuticle several hours before oviposition, but further biochemical and physiological studies are required to understand the nature of the processes involved in the decline in colour intensity with age.

Acknowledgements

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Summary

Brown eggs constitute over 99% of the UK market. Despite the successes of geneticists in selecting for high production rates with brown egg layers, variability and decline in the shell pigmentation as birds age cause economic losses.

The pigmentation of eggs is variable throughout the avian phylum and this aspect is briefly reviewed. In most species, the principal pigments found in eggshells are protoporphyrin, the immediate precursor to the red blood cell pigment, haemoglobin, and biliverdin which is the breakdown product of haemoglobin. Normally the eggshell from the domestic fowl, *Gallus domesticus*, contains mainly protoporphyrin. However, a mutation found in the Chilean Araucano fowl gives rise to blue or green eggs due to the presence of a mixture of biliverdin, its zinc chelate and protoporphyrin throughout the shell.

Recent studies confirm published research over the past 20 years that the brownness of the hen's egg is dependant on the amount of protoporphyrin adsorbed or associated with a thin layer (about 10⁻⁶m) of protein, called the cuticle, which covers the shell. Although the calcareous shell contains pigment, its contribution to the intensity of colour of the egg is negligible compared with that of the cuticle.

The protoporphyrin appears to be synthesised and secreted by the epithelial cells of the shell gland. The final coat of pigment is transferred to the cuticle several hours before oviposition. This aspect of shell pigmentation contrasts with that of the yolk which depends on the incorporation of suitable carotenoid pigments in the diets of layers.

If a hen is subjected to stress, the formed egg may be retained in the shell gland beyond its normal time of oviposition. Under these circumstances, the colour of brown eggs may be modified by a superficial coating of calcareous material secreted by the shell gland. Diseases or drugs may also cause a reduction in shell pigment.

Résumé

UNE REVUE SUR LA PIGMENTATION DE LA COQUILLE DES OEUFS (M. R. Lang et J. W. Wells)

Les oeufs bruns constituent plus de 90% du marché du Royaume-Uni. Malgré les succès des généticiens pour sélectionner pour un taux de production élevé des pondeuses à oeufs bruns, la variabilité et la diminution de la pigmentation des coquilles avec l'âge des pondeuses occasionnent des pertes économiques.

La pigmentation des oeufs est variable à travers l'ensemble du phylum aviaire et cet aspect est brièvement revu. Dans la plupart des espèces, les principaux pigments trouvés dans les coquilles sont la protoporphyrine, précurseur immédiat du pigment des globules rouges, l'hémoglobine, et la biliverdine qui est un produit de dégradation de l'hémoglobine. Normalement, la coquille de l'oeuf des volailles domestiques, *Gallus domesticus*, contient principalement de la protoporphyrine. Cependant, un mutant originaire du Chili, la poule Araucana, pond des oeufs bleus ou verts dûs à la présence d'un mélange de biliverdine, de son chélateur à base de zinc et de protoporphyrine dans toute la coquille.

Des études récentes confirment les recherches publiées au cours des vingt dernières années, selon lesquelles la teinte brune de l'oeuf de poule dépend de la quantité de protoporphyrine adsorbée ou associée avec une couche fine (environ 10^μ) de protéines, appelée la cuticule, qui recouvre la coquille. Quoique la coquille calcaire contienne du pigment, sa contribution à l'intensité de la couleur de l'oeuf est négligeable comparée à celle de la cuticule.

La protoporphyrine apparaît être synthétisée et sécrétée par les cellules épithéliales de la glande coquillière. Le revêtement final de pigment est transféré à la cuticule plusieurs heures avant l'oviposition. Cet aspect de la pigmentation de la coquille contraste avec celle du jaune qui dépend de l'incorporation de pigments caroténoïdes convenables dans les rations pour pondeuses.

Si une poule est soumise à un stress, l'oeuf formé peut être retenu dans la glande coquillière au-delà du temps normal d'oviposition. Dans ces conditions, la couleur des oeufs bruns peut être modifiée par une couche superficielle de matériau calcaire sécrétée par la glande coquillière. Les maladies ou des médicaments peuvent aussi réduire la pigmentation de la coquille.

Zusammenfassung

EINE ÜBERSICHT ÜBER DIE PIGMENTIERUNG DER EISCHALE (M. R. Lang und J. W. Wells)

Auf dem englischen Markt liegt der Anteil brauner Eier über 99%. Trotz der Erfolge der Genetiker bei der Selektion auf hohe Legeraten bei Hennen für braunschalige Eier, verursacht die Varilität und die Abnahme der Schalenfarbe mit zunehmendem Alter der Hennen wirtschaftliche Verluste.

Die Pigmentierung der Eier und ihre Variabilität kommt bei allen Geflügelarten vor und dieser Gesichtspunkt wird kurz diskutiert. Bei den meisten Arten sind die hauptsächlichsten Pigmente, die in den Eischalen gefunden werden, Protoporphyrine, die unmittelbare Vorstufe des Farbstoffs roter Blutzellen, Hämoglobin, sowie Biliverdin, das Abbauprodukt von Hämoglobin. Normalerweise enthalten die Eischalen des Haushuhns (*Gallus domesticus*) hauptsächlich Protoporphyrin. Allerdings legt eine chilenische Mutante, das Araucana-Huhn, blaue oder grüne Eier wegen der Anwesenheit einer Mischung von Biliverdin, dessen Zinkchelate und Protoporphyrin, das die Schale vollständig durchdringt.

Neuere Untersuchungen bestätigen die veröffentlichten Forschungsergebnisse der letzten 20 Jahre, daß die Braunfärbung von Hühnereiern von der Menge Protoporphyrin abhängt, die von einer dünnen Eiweißschicht (etwa 10 µm) absorbiert wurde, die als Kutikula bezeichnet wird und die Schale bedeckt. Obwohl die Kalkbestandteile der Schalen Pigment enthalten, ist dessen Beitrag zur Intensität der Schalenfarbe zu vernachlässigen, im Vergleich zu dem der Kutikula.

Das Protoporphyrin wird offenbar in den Epitelzellen der Schalendrüsen synthetisiert und ausgeschieden. Die endgültige Pigmenthülle wird mehrere Stunden vor der Eiablage auf die Kutikula übertragen. In dieser Hinsicht unterscheidet sich die Pigmentierung der Schale von der des Eigelbs, die von der Verfügbarkeit geeigneter karotinoider Farbstoffe in der Ration der Legehennen abhängt.

Wenn eine Henne Streß ausgesetzt ist, kann das sich bildende Ei in der Schalendrüse über den normalen Zeitpunkt der Eiablage hinaus festgehalten werden. Unter diesen Umständen, kann die Farbe brauner Eier verändert werden durch oberflächliche Auflagerungen von kalkhaltigem Material, das von der Schalendrüse ausgeschieden wird. Krankheiten oder Medikamente können ebenfalls zu einer Verringerung der Schalenpigmentierung führen.

Resumen

REVISION DE LA PIGMENTACION DE LA CASCARA DEL HUEVO (M. R. Lang y J. W. Wells)

Los huevos rubios constituyen más del 99% del mercado en el RU. A pesar del éxito de los genetistas en seleccionar para obtener ponedoras de huevos rubios de alto nivel de producción, la variabilidad y el descenso en la pigmentación de la cáscara conforme envejecen las aves, causan pérdidas económicas.

La pigmentación de los huevos es variable en todo el phylum aviar y este aspecto es revisado brevemente. En la mayoría de las especies, los pigmentos principales encontrados en las cáscaras de los huevos son la protoporfirina, precursor inmediato del pigmento de los eritrocitos, la hemoglobina y la biliverdina que es el producto que se origina en la descomposición de la hemoglobina. Normalmente, la cáscara del huevo de la gallina doméstica, *Gallus domesticus*, contiene principalmente protoporfirina. Sin embargo, un mutante chileno, la gallina araucana, pone huevos azules o verdes debido a la presencia de una mezcla de biliverdina, su quelato de zinc y protoporfirina en toda la cáscara.

Estudios recientes confirman la investigación publicada a lo largo de los pasados 20 años de que el oscurecimiento amarronado de los huevos de gallina depende de la cantidad de protoporfirina adsorbida o asociada con una capa delgada (alrededor de 10 µm) de proteína llamada la cutícula, que cubre la cáscara. Aunque la cáscara calcárea contiene pigmento, su contribución a la intensidad del color del huevo es escasa comparada con la de la cutícula.

La protoporfirina parece ser sintetizada y segregada por las células epiteliales del útero. La capa final de pigmentación es transferida a la cutícula varias horas antes de la puesta. Este aspecto de la pigmentación de la cáscara contrasta con el de la yema que depende de la incorporación de pigmentos carotenoides adecuados en la dieta de las ponedoras.

Si se somete a estrés a la gallina, el huevo formado puede quedar retenido en el útero durante más tiempo del que ordinariamente acontece en una postura normal. En estas circunstancias, el color de los huevos rubios puede ser modificado mediante una capa superficial de material calcáreo segregado por el útero. Las enfermedades o los medicamentos también pueden causar una reducción en la pigmentación de la cáscara.

ОБЗОР ПО ПИГМЕНТАЦИИ ЯИЧНОЙ СКОРЛУПЫ
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Коричневые яйца составляют более 90% на рынке Великобритании. Несмотря на достигнутые успехи генетиков в области отбора на высокую продуктивность яичных кур, производящих коричневые яйца, изменчивость и снижение пигментации скорлупы с возрастом птицы вызывает экономические потери. Пигментация яиц изменчива в течение всего периода кладки, и этот вопрос кратко излагается в обзоре. У большинства кроссов главными пигментами в яичной скорлупе являются протопорфилин, непосредственный предшественник пигмента красных кровяных телец, гемоглобин и биливердин, являющийся продуктом распада гемоглобина. Обычно яичная скорлупа у домашней птицы *Gallus domestic* содержит главным образом протопорфилин. Однако, чилийский мутант Араукарная птица откладывает голубые и зеленые яйца в виду наличия биливердиновой смеси, хелата цинка и протопорфина в скорлупе яиц. Последние исследования подтверждают опубликованные данные по изучению этого вопроса в течение последних 20 лет относительно того, что коричневый цвет куриного яйца зависит от количества протопорфина, адсорбированного или соединенного с тонким слоем (около 10 м) протеина, называемого кутикулой, который покрывает скорлупу. Несмотря на то, что известковая скорлупа содержит пигмент, его влияние на интенсивность окраски яйца ничтожно по сравнению с влиянием кутикулы. Протопорфин синтезируется и выделяется эпителиальными клетками скорлупной железы. Окончательное покрытие пигментом передается кутикуле за несколько часов до кладки яиц. Эта пигментация скорлупы контрастирует с пигментацией желтка, которая зависит от введения соответствующих каротиноидных пигментов в рацион несушек. Если птица подвергается стрессу, то у формирующегося яйца может наблюдаться отставание в развитии скорлупной железы. В этих условиях коричневая окраска яиц может измениться под влиянием поверхностного покрытия известковым веществом, выделенным скорлупной железой. Заболевания или лечебные препараты могут также вызвать понижение пигментации скорлупы.

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